

INVESTIGATING THE ROLE OF PUTRESCINE TRANSPORT AND BIOSYNTHESIS IN
VIBRIO CHOLERAE BIOFILM FORMATION.

A Thesis
by
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Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2016
Department of Biology

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Abstract

INVESTIGATING THE ROLE OF PUTRESCINE TRANSPORT AND BIOSYNTHESIS IN *VIBRIO CHOLERA* BIOFILM FORMATION.

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Vibrio cholerae is the causative agent of the cholera disease. The natural habitat of *V. cholerae* is brackish saltwater environments where it forms biofilms in association with copepods. Biofilms are structures composed of a matrix of exopolysaccharides, DNA, protein, and bacteria cells; biofilms provide protection from harmful environmental conditions and allow *V. cholerae* to pass through the acidic environment of the human stomach. Formation of biofilms in *V. cholerae* and other bacteria are regulated by a wide variety of environmental signals. One of these signals is polyamines. In *V. cholerae*, norspermidine and spermidine have been shown to affect biofilm formation; however, the role of putrescine synthesis and transport in regulating biofilm in *V. cholerae* has not been studied. This research focused on the only proposed putrescine transport system in *V. cholerae*, PotE, and the two putrescine biosynthesis pathways. The objectives of this study were: to characterize PotE and confirm that it is a putrescine/ornithine antiporter, to determine the effect that putrescine has on biofilm formation in *V. cholerae*, and to determine the effect that inhibiting the putrescine transport and biosynthesis pathways have on polyamine concentrations in *V. cholerae*. My results indicate that under most growth conditions tested putrescine does not appear to have an impact on biofilm formation in *V. cholerae*. There is one exception, wild type *V. cholerae* grown at a low pH in the

presence of excess ornithine forms less biofilms than wild type not supplemented with ornithine or $\Delta potE$ mutants with or without ornithine. Additionally, I was able to confirm that PotE is a putrescine/ornithine antiporter using High Performance Liquid Chromatography. This is the first confirmed putrescine transporter in *V. cholerae*. Additionally, this data also indicates a potential link between cadaverine and putrescine. When ornithine to putrescine biosynthesis is inhibited, intracellular levels of cadaverine increase significantly. As cadaverine is linked to stress response, the increase in cadaverine indicates that when ornithine cannot be decarboxylated into putrescine this signals to the bacteria that it is in a stressful growth condition. This research provides insight into the life cycle of *V. cholerae* by identifying polyamine transport pathways and growth conditions under which they are utilized.

Acknowledgments

I would like to acknowledge the Appalachian State University Office of Student Research for contributing to the funding for this research and Patrick Woster from the Medical University of South Carolina for providing the DFMO used in this study. This research was also supported in part by grant #AI096358 from the National Institute of Allergy and Infectious Diseases awarded to Ece Karatan. I would like to thank the members of the Karatan lab, past and present, for support of this research. Thank you to the members of my committee for being mentors, friends, and role models during my time at Appalachian State. A special thank you to my advisor, Dr. Ece Karatan, for providing support above and beyond expectation, being an exceptional mentor when I most needed it, and believing in me and my abilities.

Dedication

This work is dedicated to my mother, Donna Isenhowe; without her relentless encouragement of my studies and unending support I would not be where I am today. This work would not have been possible without her support, love, and guidance. To my late father, Troy Isenhowe, who always believed in me and my ability to be better. Finally, to my fiancée, Garrett Ballard, who has read enough drafts of this work to classify as my biggest fan.

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Introduction

Vibrio cholerae is an aquatic, Gram negative pathogenic bacterium that can infect and colonize the human intestinal tract. The natural habitat of *V. cholerae* is brackish or saltwater environments but it is able to colonize freshwater as well. Humans usually become infected with *V. cholerae* when they ingest contaminated food or water. The bacterium is the causative agent of the diarrheal disease known as cholera. Cholera can be fatal within 24 hours if not treated; the main treatment is rehydration therapy. In developing countries, cholera still poses a significant health risk due to poor sanitation and poverty. The symptoms of the disease are caused by the cholera toxin, which *V. cholerae* secretes during infection.

In its natural environment, planktonic, or individual, *V. cholerae* is susceptible to environmental stressors, which it deals with by forming associations known as biofilms. Biofilms are generally formed in association with surfaces, particularly the exoskeleton of copepods (Huq et al., 1983). Water contaminated with colonized copepods is a major source of human infection and simple filtration which removes zooplankton can significantly decrease the rate of human infection (Colwell et al., 2003; Huq et al., 1996). Ability to form a biofilm is a mechanism of survival for bacteria. Bacteria in an aquatic environment are rarely found in their free swimming, planktonic state and instead form attached associations or biofilms (Costerton et al., 1987; Lee et al., 2009; Moorthy & Watnick, 2004; Watnick & Kolter, 1999). Biofilms provide bacteria such as *V. cholerae* protection from environmental stresses such as gastric acid, which allows *V. cholerae* to move through the acidic human stomach and colonize the more pH neutral small intestines. Planktonic *V. cholerae* cannot survive the acidic environment of the stomach, and therefore the bacteria rely on biofilms to carry out its infection cycle. Once in the intestines, *V. cholerae* will emerge from the biofilm state to the planktonic state. *V. cholerae* in the planktonic

state will further colonize the intestines and begin to release cholera toxin; planktonic *V. cholerae* is the only state that can release the cholera toxin.

In *V. cholerae*, growth in a biofilm has been shown to induce a hyperinfectious phenotype, an increased ability to persist in environment, and an increased ability to infect a host (Faruque et al., 2006; Tamayo, Patimalla, & Camilli, 2010). It has been proposed that biofilms in aquatic environments provide protection from potential hypo-osmotic stress that a lone bacterium would face, thereby making a biofilm state more advantageous (Faruque et al., 2006). Biofilms usually consist of a multitude of bacteria in a matrix of DNA, protein, and exopolysaccharides. In the majority of cases this matrix accounts for up to 90% of the dry weight of a biofilm, with cells only making up 10% of the weight (Flemming & Wingender, 2010). The extracellular matrix provides a number of advantages to the biofilm, including: aiding in bacterial adhesion, as a nutrient source, retention of water, genetic sharing, and general protection from stressors (Flemming & Wingender, 2010).

Biofilm formation occurs in multiple steps (Figure 1) (Watnick & Kolter, 2000). It starts with free swimming planktonic bacteria approaching and attaching to or associating with a surface. Initially, this attachment is transient and reversible for the bacteria. This first surface attachment initiates the bacteria becoming immobilized and they then move into a more permanent surface attachment. Cells in this monolayer specialize so as to maintain their surface attachment (Moorthy & Watnick, 2004). A bacterial cell will form associations with other bacterial cells at this point, leading to the formation of microcolonies within the biofilm, these microcolonies then begin to form towers (Watnick & Kolter, 2000). At this point a three dimensional shape emerges formed out of pillars of bacteria; this is the mature biofilm (Karatan & Michael, 2013; Watnick & Kolter, 2000; Watnick & Kolter, 1999). Various environmental

signals signal the transitions from a planktonic cell to life in a biofilm (Moorthy & Watnick, 2004). One such signal is the small group of molecules known as polyamines (Karatan, Duncan, & Watnick, 2005).

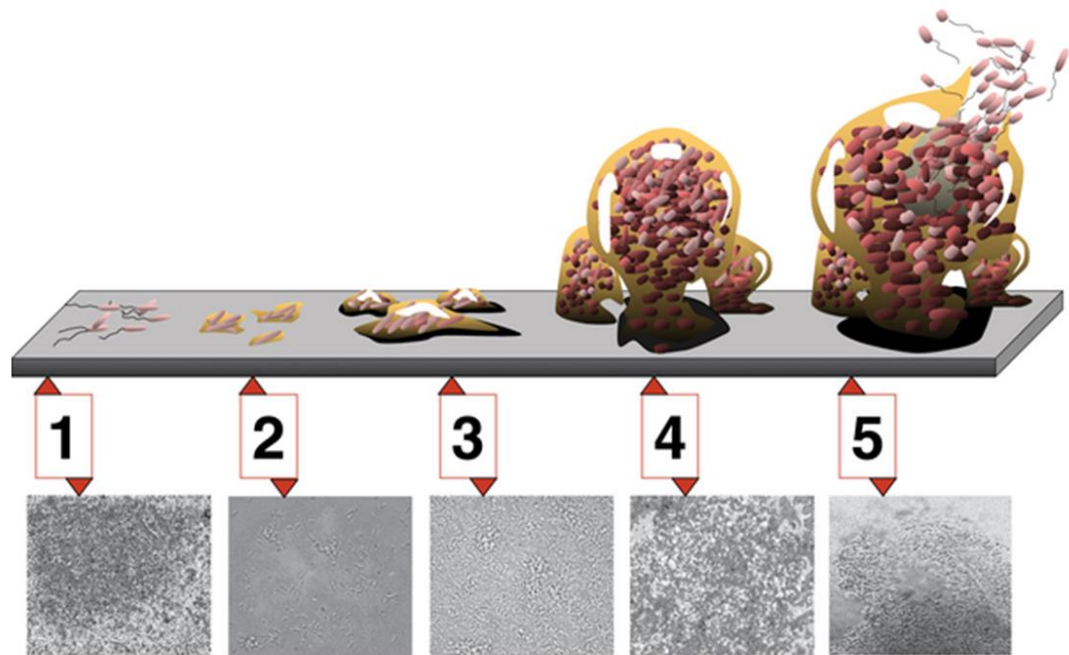


Figure 1: Stages of biofilm formation: Adapted from Monroe 2007 (Monroe, 2007). The 5 stages of biofilm development. Image credit D. Davis 1) Reversible initial attachment to the surface. 2) Permanent attachment to the surface. 3) Early development of vertical colonies. 4) Multiple biofilm towers developing. 5) Mature biofilm with fully developed towers able to disperse free swimming planktonic organisms.

A polyamine is a small molecule found in most eukaryotic and prokaryotic cells. While small, polyamines are involved in a variety of cellular functions (Igarashi & Kashiwagi, 2010b).

All polyamines are positively charged at physiological pH and are derived from amino acids (Michael, 2016). They also have a hydrocarbon background and multiple amine groups.

Polyamines are found in relatively high abundance in living systems (Munro, Bell, & Lederman,

1974; Tabor & Tabor, 1984). The most commonly found polyamines are putrescine and spermidine; however, the type and composition of polyamines varies between species (Morgan, 1999; Tabor & Tabor, 1985). One pathway of biofilm formation in *Vibrio cholerae* is regulated by norspermidine (Karatan et al., 2005; Karatan & Watnick, 2009; Lee et al., 2009; Parker, Pendergraft, Sobieraj, McGinnis, & Karatan, 2012). Norspermidine is a triamine which can stimulate biofilm formation in *Vibrio cholerae* (Karatan et al., 2005; Karatan & Watnick, 2009). At concentrations of 100 μ M, norspermidine leads to a threefold increase in biofilm formation in *Vibrio cholerae* (Karatan et al., 2005).

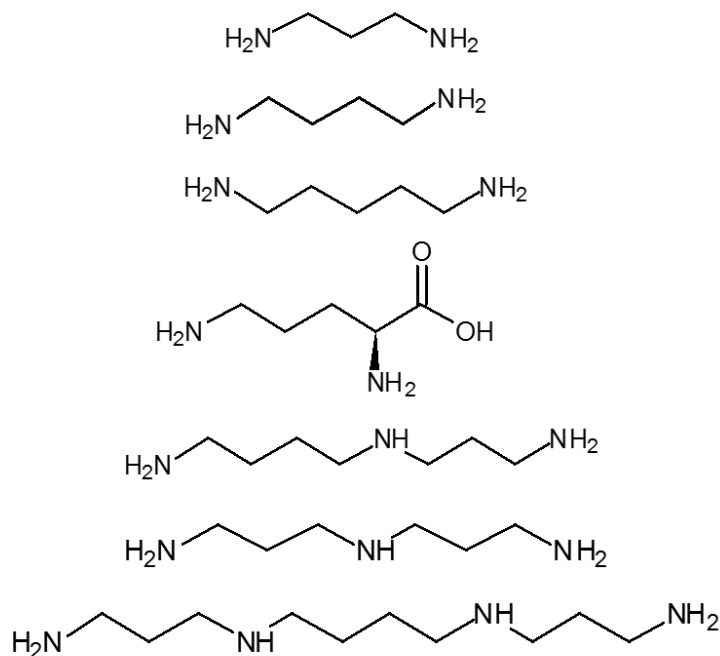


Figure 2: Chemical structures of polyamines. This shows the chemical structure of Diaminopropane, Putrescine, Cadaverine, Ornithine, Spermidine, Norspermidine, and Spermine in respective order.

Since it has been shown that norspermidine can effect biofilm formation, it was of interest to investigate other polyamines for their potential effects on biofilm formation in *V.*

cholerae. Putrescine, a small polyamine, is known to be present intracellularly but prior to this research little was known about the synthesis and transport of this polyamine in *V. cholerae*. Putrescine (1,4-diaminobutane) is a small, cationic, foul smelling polyamine originally identified by Ludwig Brieger from cadavers in 1885 and it is required for optimum growth in many cells (Cunningham-Rundles & Maas, 1975; Moore & Boyle, 1991). The goal of this project was to learn if putrescine, like norspermidine, regulates biofilm formation in *V. cholerae*. To do this, I investigated the only proposed putrescine transport system in *V. cholerae* and the two proposed biosynthesis pathways.

As putrescine biosynthesis and transport has not been investigated in *V. cholerae* the knowledge of the pathways come from other bacteria, namely *Escherichia coli*. Part of this research was to investigate if the pathways were conserved between *E. coli* and *V. cholerae*. I have found using comparative genomic analysis that there is one possible putrescine transport protein in *V. cholerae* known as PotE. *E. coli* has other putrescine importers including: PuuP, PlaP, PotFGHI none of which are encoded in the *V. cholerae* genome. There are two pathways of putrescine biosynthesis proposed in *V. cholerae*; they are the same as those in *E. coli*. In *E. coli* putrescine is synthesized through two pathways (Morris & Pardee, 1966). Both pathways appear to synthesize putrescine with equal efficiency but under different conditions (Morris & Pardee, 1966). One pathway uses the enzyme ornithine decarboxylase, which is encoded by the *speC* gene in *E. coli*. The *speC* gene is also present in the MO10 strain of *V. cholerae* that was used for this research. In this pathway, ornithine is decarboxylated by ornithine decarboxylase into putrescine (Moore & Boyle, 1991; Song et al., 2010; Tabor & Tabor, 1985). Ornithine to putrescine is the most direct pathway of putrescine synthesis in *E. coli* (Morris & Pardee, 1966). During the decarboxylation of ornithine a CO₂ molecule is released, yielding putrescine (Morris

& Pardee, 1966). This is also the preferred route when cells are grown in a minimal media, as it is energetically advantageous (Morris & Koffron, 1969). In some organisms, arginine can be converted into ornithine which is then converted to putrescine as outlined above; however, this is not the case in *E. coli* (Morris, Wu, Applebau.D, & Koffron, 1970). Instead, a second pathway of arginine conversion exists in bacteria and plants. This pathway converts arginine to agmatine via the removal of the arginine carboxyl group as CO₂ via the arginine decarboxylase enzyme, which is encoded by the *speA* gene (Moore & Boyle, 1991; Morris & Pardee, 1966; Morris et al., 1970; Satishchandran & Boyle, 1986; Song et al., 2010; Tabor & Tabor, 1985). Agmatine is then hydrolyzed into putrescine via the release of urea; this hydrolysis reaction is carried out by the enzyme agmatine uerohydrolase encoded by the *speB* gene (Szumanski & Boyle, 1990). I focused on the *speB* gene found in the MO10 strain of *V. cholerae* in this research to eliminate the last step of this putrescine biosynthesis pathway. The arginine to putrescine pathway is preferred when cells are grown in media supplemented with arginine.

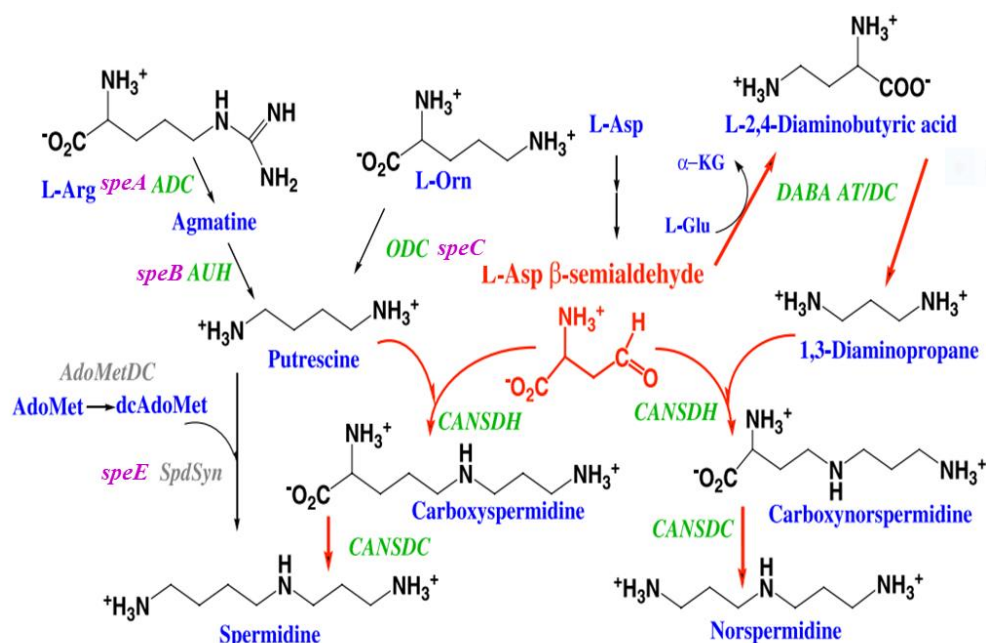


Figure 3: Polyamine biosynthesis pathways. Adapted from Lee, Sperandio et al. 2009 (Lee et al., 2009). Green enzymes are present in *Vibrio cholerae* while grey enzymes are not. Purple text represents gene names. Red arrows show alternative pathways. Chemical names are listed in blue.

The putrescine/ornithine antiporter *potE* was first characterized and sequenced in *E. coli* with its operon partner *speF* in 1991 and it is found in many Gram-negative bacteria (Kashiwagi et al., 1991; Minchin & McCoubrie, 2004). In *E. coli*, uptake of putrescine by PotE is dependent on membrane potential while excretion is dependent on the putrescine-ornithine antiporter activity (Kashiwagi et al., 2000; Kashiwagi, Shibuya, Tomitori, Kuraishi, & Igarashi, 1997). The PotE transporter imported less putrescine than the PotABCD and PotFGHI when grown in *E. coli* K-12 in low osmolarity media (Pistocchi et al., 1993), but it is not known if this behavior is conserved in other media or other organisms, such as *Vibrio cholerae*. PotABCD is a spermidine preferential transporter that can, under certain laboratory conditions, import putrescine although it is not known if this is a frequently used pathway. PotFGHI is a putrescine preferential importer

that is not present in *V. cholerae*. The expression of the *potE* operon is repressed at neutral extracellular pH (Kashiwagi et al., 1997). PotE is inducible at low pH in the presence of excess ornithine while lack of ornithine inhibits putrescine export. Ornithine levels below 10 μ M led to ornithine not being imported by PotE (Kashiwagi et al., 1997). The inhibition of the *cadBA*, which encodes the cadaverine importer CadB, operon also induces *potE* (Igarashi & Kashiwagi, 2010a; Soksawatmaekhin, Kuraishi, Sakata, Kashiwagi, & Igarashi, 2004; Tomitori, Kashiwagi, & Igarashi, 2012). PotE and CadB are similar importers just as putrescine and cadaverine are similar molecules. CadB is a cadaverine-lysine antiporter which shows similarities to the putrescine-ornithine antiporter PotE (Soksawatmaekhin et al., 2004; Tomitori et al., 2012). The *cadBA* system is induced at low pH and it is believed to be a mechanism for *E. coli*, and potentially other bacteria, to respond to acidic conditions (Küper & Jung, 2006; Meng & Bennett, 1992; Watson, Dunyak, Rosey, Slonczewski, & Olson, 1992). In *Vibrio vulnificus* and *V. cholerae* low pH triggers the production of cadaverine, the cadaverine is then excreted and neutralizes low environmental pH and protects the cells from acid stress (Kang, Kim, & Lee, 2009; Merrell & Camilli, 1999).

There is previous research to indicate that putrescine effects biofilm formation in other bacteria. *Yersinia pestis* is the causative agent of plague, and is generally passed to humans via flea bites. It was found that polyamines, specifically putrescine, were necessary for the formation of biofilms in *Yersinia pestis* (Patel et al., 2006). The *speA* and *speC* pathways lead to the formation of putrescine (Figures 3, 25), which is also a vital polyamine precursor to other important polyamines such as spermidine in many bacteria and other organisms (Morris & Pardee, 1966; Tabor & Tabor, 1984, 1985). When the biosynthetic pathways of arginine decarboxylase (*speA*) and ornithine decarboxylase (*speC*) which make putrescine in *Yersinia*

pestis were deleted, biofilm formation was impaired (Patel et al., 2006). Biofilm formation was lower in single mutant strains lacking either *speA* or *speC* and eliminated in double mutant strains lacking both *speA* and *speC* (Patel et al., 2006). The single mutant strains suggested that there is a threshold of putrescine required for biofilm formation. When both *speA* and *speC* were deleted there was a greater than 99% reduction in putrescine levels and no detectable levels of putrescine or spermidine in the mutant cells (Patel et al., 2006).

The aim of this project was multifold, but overall I wanted to establish the impact that putrescine had on biofilm formation. Initially, I wanted to confirm that PotE is a putrescine/ornithine antiporter using HPLC analysis and establish if the deletion of *potE* had any effect on biofilm formation. Secondly, I wanted to inhibit the biosynthesis pathways present in *V. cholerae* to gauge the effects on biofilm formation and to better control the putrescine inputs to the bacteria. This was done in two ways; via the deletion of the *speB* gene and the inhibition of ornithine decarboxylase, the product of the *speC* gene, via a chemical known as DFMO.

I was able to confirm that PotE is a putrescine/ornithine antiporter using HPLC analysis to measure the intracellular and extracellular polyamine levels in wild type and $\Delta potE$ cells grown at low pH with and without ornithine supplementation. This is the first confirmation of a putrescine transport system in *V. cholerae*. Biofilms formed by the mutant strains were generally not significantly different from that of wild type. Addition of DFMO increased biofilm formation overall, but there was no significant difference between strains. One growth condition did show significant impact on biofilm formation. When wild type was grown in a low pH environment with excess ornithine in the growth media the amount of biofilm formed decreased significantly from wild type without additional ornithine or the $\Delta potE$ mutant. Overall the results of my study show how *V. cholerae* uses putrescine under certain environmental conditions as a negative

regulator for biofilm formation and inhibition of ornithine decarboxylation/PotE function is viewed by the cell as a stressful situation on par with acid stress.

Materials and Methods

Media

Bacteria were typically grown on Luria-Bertani (LB) broth or solid LB agar plates containing 1.5% agar. (Bertani, 1951; Bertani, 2004). Tryptone broth was used only when specified. LB broth contains 1% tryptone, 0.5% yeast extract, and 85mM NaCl. Tryptone broth contains 1% tryptone and 85mM NaCl. Antibiotics were added to the cultures for bacterial selection. Streptomycin was used for *V. cholerae* selection and ampicillin was used for *E. coli* selection. Antibiotics were used at a concentration of 100µg/mL unless otherwise specified. *V. cholerae* was normally grown at 27°C while *E. coli* was grown at 37°C. The only time *V. cholerae* were grown at 37°C was during conjugation with *E. coli*. All enzymes used were purchased from New England BioLabs (Ipswich, MA)

For some experiments, the pH of the growth media was adjusted. When this was necessary an Accumnet AB15 Plus pH Meter (ThermoFisher, Waltham MA) was used to adjust 250mL of LB or Tryptone broth using concentrated NaOH or HCl. The media was then autoclaved and the pH was checked using MColorpHast pH indicator strips (EMD Millipore, Billerica MA). Overnight and day cultures were grown in regular media that did not have the pH altered.

Bacterial Strains

The strains, plasmids, and primers used throughout this study are listed in Tables 1, 2, and 3 respectively. All *V. cholerae* used in this study was the serotype 0139 strain MO10 in which the *lacZ* gene was disrupted by inserting a construct containing a promoter fusion of the *vpsL* promoter to the *E. coli lacZ* gene. All *E. coli* strains used or generated in this course of study had a background of DH5 α , DH5 α λ pir, or SM10 λ pir.

Table 1: Bacterial Strains

Strain	Genotype	Reference/Source
<i>E. coli</i>		
DH5 α	F- Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Invitrogen
DH5 α λ pir	<i>supE44, \Delta lacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, \lambda pir</i>	Hanahan 1983
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu\lambda pirR6K; Km^R</i>	Miller and Mekalanos 1988
AK523	DH5 α :: Δ <i>potE</i> , Amp ^R	This study
AK530	DH5 α λ pir:: Δ <i>potE</i> , Amp ^R	This study
AK533	SM10 λ pir:: Δ <i>potE</i> , Amp ^R	This study
AK592	DH5 α :: Δ <i>speB</i> , Amp ^R	This study
AK597	DH5 α λ pir:: Δ <i>speB</i> , Amp ^R	This study
AK600	SM10 λ pir:: Δ <i>speB</i> , Amp ^R	This study
	DH5 α :: Δ <i>speC</i> , Amp ^R	This study
	DH5 α λ pir:: Δ <i>speC</i> , Amp ^R	This study
	SM10 λ pir:: Δ <i>speC</i> , Amp ^R	This study
<i>V. cholerae</i>		
PW357	MO10 <i>lacZ::vpsLp lacZ</i> , Sm ^R	Haugo and Watnick 2002
AK536	PW357:: Δ <i>potE</i> , Sm ^R	This study
AK616	PW357:: Δ <i>speB</i> , Sm ^R	This study
AK630	PW357:: Δ <i>speB</i> Δ <i>potE</i> , Sm ^R	This study

Generation of the *potE*, *speB*, and *speC* deletion constructs:

Previous studies in the Karatan lab have constructed in-frame deletions of other *V. cholerae* genes, these methods were adapted from their work (McGinnis et al., 2009; Sanders, 2015). Primers (Table 3) were developed for the deletion of the *potE* gene using a *potE* MO10 gene sequence downloaded from The Broad Institute (www.broadinstitute.org). Vector NTI (ThermoFisher, Waltham MA) was used to identify the reading frame. For the deletion of *speB*, the *speB* MO10 gene sequence was downloaded from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) and Vector NTI was used to identify the correct reading frame. The gene sequence of *speC* MO10 was downloaded from GenBank and Vector NTI was used to identify the correct reading frame. Primers synthesis for $\Delta potE$ was performed by Eurofins MWG Operon (Huntsville AL) while primer synthesis for $\Delta speB$ and $\Delta speC$ was performed by Invitrogen (ThermoFisher, Waltham MA).

Primers were combined in a series of PCR reactions using Q5 polymerase (New England BioLabs, Ipswich MA) to make one fragment for use in deletion of the *potE*, *speB*, or *speC* genes. Initially, fragments containing approximately 350-450bp upstream and downstream of the *potE*, *speB*, and *speC* genes were amplified by PCR using *V. cholerae* chromosomal DNA. To fuse the fragments together, internal primers were generated with overlapping regions of complementary DNA. Once these fragments were combined in a PCR reaction the overlapping segments allowed the 3' end of the top strand to anneal onto the 3' end of the bottom strand thereby splicing these regions together. Splicing by overlap extension of the regions yielded an approximately 700-1000bp segment that generated an in-frame deletion in the gene of interest (990bp for *potE*, 717bp for *speB*, 863bp for *speC*) (Figure 4).

After PCR amplification and purification, adenines were added to the 3' ends of the PCR product to allow for pCR2.1 TOPO cloning. Ten μL of the purified PCR product was added to a mixture of 28.5 μL of water, 5 μL of 5x *OneTaq* polymerase buffer, 1 μL of 10mM dATP, and 0.5 μL of *OneTaq* polymerase. This mixture was allowed to incubate for 10 minutes at 68°C, as specified by the *OneTaq* instruction guide. This product was then cloned into pCR2.1-TOPO following manufactures recommendations (Invitrogen, Carlsbad CA) with an incubation time of 30 minutes. The pCR2.1 with inserted ΔpotE , ΔspeB , or ΔspeC was then electroporated into DH5 α using a BIO-RAD MicroPulser (Hercules CA) at 1.8kV. The transformed cells were allowed to recover in warmed Super Optimal broth with Catabolite repression media (also known as SOC, composed of 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM glucose) for one hour at 37°C with shaking at 200rpm. The recovered cells were then plated on LB-ampicillin (100 $\mu\text{g}/\text{mL}$) agar plates that had been coated with 20 μL of X-gal (30mg/mL in dimethyl sulfoxide) to allow for blue/white screening.

Blue/white screening was used to select for bacterial colonies carrying the inserted plasmid. White cells were chosen and patched onto LB-ampicillin plates coated with X-gal. After growth, a colony PCR was performed to verify the presence of the insert. A swab of the patched colony was suspended in 100 μL of water and heated at 95°C for 5 minutes in order to lyse the cell. The water containing the cell lysate was spun at 8k for 5 minutes in order to remove cellular debris, 2 μL of the lysate water was then used in a PCR reaction with 16.4 μL of water, 5 μL of 5X *OneTaq* standard reaction buffer, 0.5 μL 10mM dNTP, 0.5 μL of each outside primer (at 10 μM concentration), and .125 μL of *OneTaq* DNA polymerase. To ensure a consistent amount of each reagent was added, a master mix was made with all of the above ingredients for $n+1$ reactions (n =number of colonies screened). The cellular lysate was added to each individual

PCR reaction. PCR conditions were as follows: initial denaturing for 30 seconds at 94°C, then 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 30s, and a final extension at 72°C for 5 minutes.

After verification of the insert, the plasmid was isolated using a Promega Wizard Plus SV Miniprep Purification System (Promega Corporation, Fitchburg WI) and sent for sequencing to ensure correct construction of the in-frame deletion construct. Once correct assembly was verified, the plasmid was digested with XhoI and SpeI enzymes (New England BioLabs, Ipswich MA) and ran out on a gel. The $\Delta potE$, $\Delta speB$, or $\Delta speC$ construct was isolated via gel purification using a Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK) and then ligated into a pWM91 plasmid digested with the same enzymes and gel-purified using ElectroLigase (New England BioLabs, Ipswich MA). This plasmid was then electroporated into DH5α λ pir and a colony PCR was performed to verify the insert. The plasmid was then extracted from DH5α λ pir via a Promega Wizard Plus SV Miniprep kit which would leave it supercoiled for electroporation into SM10 λ pir. SM10 λ pir is a strain of *E. coli* that will conjugate readily with *V. cholerae*.

PW357 was used for the conjugation with SM10 λ pir via SacB counter selectable mutagenesis (Metcalf et al., 1996). PW357 is an 0139 MO10 strain of *V. cholerae* that has the *vpsL* promoter fused to the *lacZ* gene (Haugo & Watnick, 2002). *V. cholerae* and the donor SM10 λ pir containing one of the deletion plasmids (pWM91:: $\Delta potE$, pWM91:: $\Delta speB$, or pWM91:: $\Delta speC$) were streaked onto new plates containing either streptomycin (for *V. cholerae*) or ampicillin (for *E. coli*) and incubated at 37°C overnight. After growth, LB plates with no added antibiotics were used to streak both the donor and recipient in a crisscross fashion which would allow for contact between the bacterial strains; this was allowed to grow overnight to

allow for conjugation to occur. The following day, half of the growth was streaked onto a new LB plate for isolation, this LB plate contained streptomycin (100µg/mL) and ampicillin (50µg/mL) to select for only those *V. cholerae* colonies that had successful crossover events; this was incubated overnight at 37°C. From there, four single colony isolates were chosen and re-streaked on streptomycin and ampicillin selection plates for purification and incubated overnight at 37°C. Following this, four colonies were streaked on LB plates with no antibiotic to allow for a second recombination event which would remove the unneeded ampicillin resistance and *sacB* genes encoded by the pWM91 plasmid; this was once again incubated overnight at 37°C. After incubation, eight colonies were selected and streaked for isolation onto sucrose plates, and incubated for two days at room temperature. Sucrose plates were made by combining 5g of yeast, 10g of tryptone, 15g agar, and 667mL of water; this mixture was then autoclaved and when cool to touch 333mL of warmed 30% filter sterilized sucrose was added. Bacteria were grown on sucrose plates to ensure that they would keep the insert. After two days of growth, 80-100 colonies were patched on two sets of plates, on streptomycin plates and then immediately on streptomycin (100µg/mL) and ampicillin (50µg/mL) plates. Colonies that only grew on the streptomycin plates and not the streptomycin and ampicillin plates underwent a colony PCR to verify the $\Delta potE$ insert using PA283 and PA302, the $\Delta speB$ insert was verified by use of PA307 and PA310, and $\Delta speC$ was verified by use of PA317 and PA318.

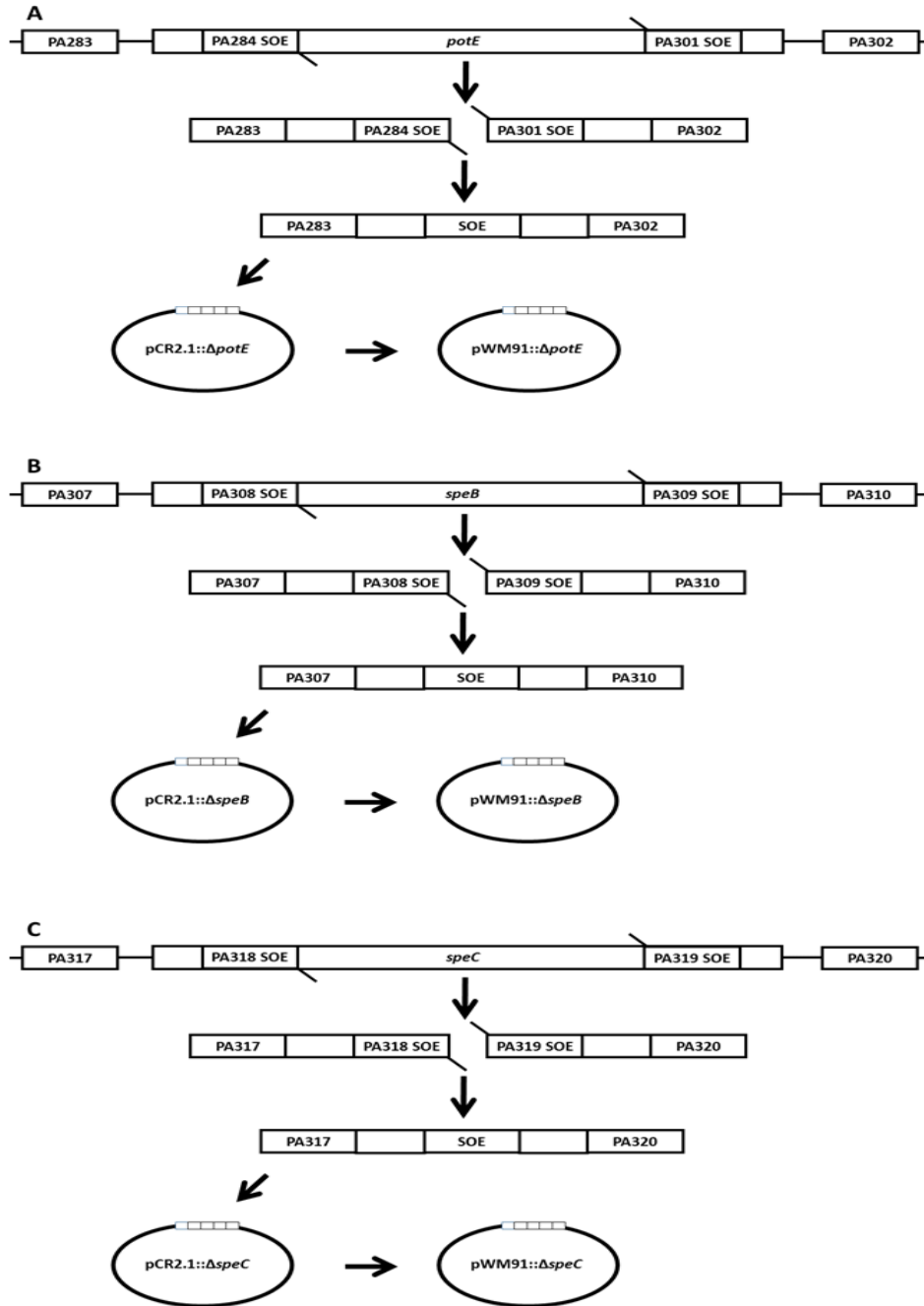


Figure 4: Construction of the pJMI2, pJMI4, and pJMI6 plasmids containing a deletion of one of the genes of interest in *Vibrio cholerae*. (A) the pJMI2 plasmid was used to delete the *potE* gene. (B) The pJMI4 plasmid was used to delete the *speB* gene. (C) The pJMI6 plasmid was used to delete the *speC* gene. Diagonal tags indicate the location of the SOE tag and where the deletion construct will splice together

Table 2: Plasmids

Plasmid	Genotype	Reference/source
pCR2.1-TOPO	Plasmid for TOPO cloning, Ap ^R	Invitrogen
pWM91	<i>oriR6K</i> , <i>lacAα</i> , <i>sacB</i> , homologous recombination plasmid Ap ^R	Metcalf et al. 1996
pJMI1	pCR2.1:: <i>ΔpotE</i> , carrying an in-frame deletion of <i>potE</i>	This study
pJMI2	pWM91:: <i>ΔpotE</i> , carrying an in-frame deletion of <i>potE</i>	This study
pJMI3	pCR2.1:: <i>ΔspeB</i> , carrying an in-frame deletion of <i>speB</i>	This study
pJMI4	pWM91:: <i>ΔspeB</i> , carrying an in-frame deletion of <i>speB</i>	This study
pJMI5	pCR2.1:: <i>ΔspeC</i> , carrying an in-frame deletion of <i>speC</i>	This study
pJMI6	pWM91:: <i>ΔspeC</i> , carrying an in-frame deletion of <i>speC</i>	This study

Table 3: Primers

Primer	Description	Sequence
PA283	Forward primer for upstream fragment of <i>potE</i> deletion	CGGTAATGAACCCACAAGAAGC
PA284	Reverse primer for upstream fragment of <i>potE</i> + SOE deletion	TTACGAGCGGCCGCAGCCCGATCCCATCATGT TAAC
PA301	Forward primer for downstream fragment of <i>potE</i> + SOE deletion (2)	TGCGGCCGCTCGTAAGCTGCGCTGGGCGTGAT GC
PA302	Reverse primer for downstream fragment of <i>potE</i> deletion (2)	GTAACCGTCGGCCCTGGCTTC
PA303	Forward midsequence primer for sequencing of gene construct	AAGGTCAACAGCGCAGCA
PA304	Forward midsequence primer for sequencing of gene construct	TGCTGCGCTGTTGACCTT
PA307	Forward primer for upstream fragment of <i>speB</i> deletion	GACACGGTTGAAGATATGATG
PA308	Reverse primer for upstream fragment of <i>speB</i> + SOE deletion	TTACGAGCGGCCGCACATATCCAAAGGCACG CC
PA309	Forward primer for downstream fragment of <i>speB</i> + SOE deletion	TGCGGCCGCTCGTAAATCCTGAAAATCATCCG C
PA310	Reverse primer for downstream fragment of <i>speB</i> deletion	TGGACGGTTACACCAACTACAATG
PA317	Forward primer for upstream fragment of <i>speC</i> deletion	CTGCTCCTACCGAAACTGAGT
PA318	Reverse primer for upstream fragment of <i>speC</i> + SOE deletion	TTACGAGCGGCCGCAGCACACAGCAACAAAG ATC
PA319	Forward primer for downstream fragment of <i>speC</i> + SOE deletion	TGCGGCCGCTCGTAAATGAACCCACAAGAAG CG
PA320	Reverse primer for downstream fragment of <i>speC</i> deletion	GCATAATGATGCCCGATCCCA

Polyamine extraction

To further identify the role of PotE, SpeB, and SpeC on uptake and synthesis of putrescine, both intracellular and extracellular polyamines were extracted. Polyamine extraction was adapted from previously described work (McGinnis et al., 2009; Morgan, 1998; Parker et al., 2012).

Cellular polyamine extraction

LB media was used for the growth of the bacteria strains. Overnight cultures with 2mL of LB and 2 μ L of streptomycin (stock concentration 100 mg/mL) inoculated with bacteria were grown for 16-18h and then 400 μ L of the overnight was added to a fresh 20mL culture. The 20mL cultures were grown at 27°C until mid-log phase as determined by optical density for about 4-5 hours and then harvested. Bacteria were pelleted at 5000 rpm and the pellet was washed and resuspended with 5mL of 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4); this step was repeated once and then bacteria were resuspended in 1mL of 1x PBS before being pelleted a third time. After pelleting, the 1x PBS was removed and the pellets were weighed and resuspended in water at 10 μ L per mg of cell weight. Cells were then lysed using a Branson SFX150 Sonicator (Branson Ultrasonics Danbury CT) and cellular debris was removed with centrifugation for 10 minutes at 5000rpm. Cellular proteins and DNA were precipitated by addition of 50% trichloroacetic acid (TCA) which was then centrifuged to pellet the unwanted material; the resulting supernatant contained the polyamines and was removed by decanting. This supernatant was ready for benzylation

Media polyamine extraction

One mL of supernatant was taken from each cellular extract after the initial spin of 5 minutes at 5000rpm. After pelleting, the supernatant was decanted into a new tube and spun again for 5 minutes at 8000 rpm to remove any remaining cells. The supernatant was removed and cell pellets were discarded. This supernatant was then treated with 50% TCA to remove any proteins and transferred into a final tube; the media containing polyamines was then ready for benzoylation.

Benzoylation

Benzoylation was performed as previously described (McGinnis et al., 2009; Morgan, 1998; Parker et al., 2012), with the amount of chloroform transferred from each tube reduced slightly to avoid contamination with the aqueous layer. For benzoylation, 500 μ L of sample derived from polyamine extraction was added to individual benzoylation tubes. With every experimental sample set a polyamine standard consisting of 495 μ L of sterile water and 5 μ L of a 10mM polyamine mixture (putrescine, diaminopropane, cadaverine, spermidine, and norspermidine) was added to a benzoylation tube to equal 500 μ L total volume and a final concentration of 0.1mM of polyamines. 2mL of 2M NaOH was added to each sample tube, this was vortexed for one minute before addition of 20 μ L of 25% benzoyl chloride. The mixture was again vortexed for at least one minute and then incubated for one hour at room temperature with shaking at 150rpm. Benzoylated polyamines were isolated with a 1mL chloroform extraction done two times per sample, after each isolation 800 μ L of chloroform was removed to a clean collection tube (1600 μ L total per sample). The samples were then washed with 1mL of sterile water and 1300 μ L of chloroform was removed into a final tube. The chloroform was evaporated

in a chemical hood to exact dryness and the benzoylated polyamines were then resuspended with 100 μ L mobile phases as used for HPLC (60% methanol/40% water).

HPLC Analysis

As previously described (Sanders, 2015) High Performance Liquid Chromatography analysis was done using a Waters 1525 Binary Pump with a Waters 2487 Dual Wavelength Absorbance Detector set at 254nm (Waters Corporation, Milford MA). A Phenomenex SphereClone 5 μ m ODS column (80 Å LC column 250 x 4.6 mm) was fitted on the HPLC (Phenomenex, Torrance, CA). For each run 40 μ L of sample was injected into the HPLC. Each sample was ran using a gradient of 45-60% methanol in water for 30 minutes followed by a 10 minute equilibration of 45% methanol in water.

Biofilm Assays

Biofilm assays were adapted from previously described work (Karatan et al., 2005). Bacteria were grown on LB agar plates overnight at 27°C. Isolated colonies were selected and put into an overnight of 2mL LB broth and 2 μ L of streptomycin. After 16-18 hours of growth 40 μ L of overnight culture was added to 2mL of fresh LB broth and 2 μ L streptomycin. This culture was grown to an OD₆₅₅ of between 0.3 and 0.4. Borosilicate tubes were inoculated with LB broth and a small amount of the bacterial culture to make a final OD of cells in the tube 0.02 in a final volume of 300 μ L. Biofilm cultures were then grown at 27°C for 24 hours. During analysis, planktonic cells were removed, 150 μ L were used to identify planktonic OD and the rest was discarded. Biofilm cells were then washed once with 300 μ L 1x PBS. The biofilm was then resuspended in 300 μ L of 1x PBS by vortexing with glass beads to make a homogenized mixture. 150 μ L of homogenized biofilm was used to determine the OD of the sample. An iMark

Microplate Reader (Bio-Rad, Hercules CA) sat at 655nm was used to quantify optical density of planktonic and biofilm cells. This was done in triplicate for technical and biological replicates. Biofilms were performed with at least three biological replicates. Two tailed *t*-test were performed in Microsoft Excel. Averages of biological replicates were taken and two conditions were compared.

Results

Construction of the $\Delta potE$ single mutant

To study the role of PotE in *V. cholerae*, I designed primers that would make a deletion within the *potE* gene. A region approximately 435bp upstream and 540bp downstream of the *potE* gene was amplified with PCR using the primers listed in Table 3. Primers PA283 and PA284 were used to amplify the upstream portion of the *potE* gene and primers PA301 and PA302 were used to amplify the downstream portion. PCR products were run on a 1% agarose gel to verify the correct size fragments (Figure 5).

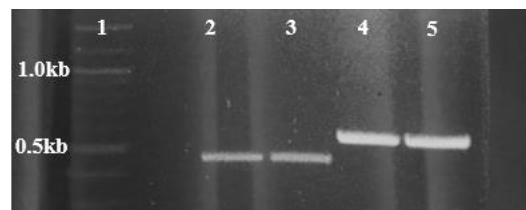


Figure 5: Initial amplification of the upstream and downstream regions of *potE*. This image represents both the upstream region at 435bp amplified by PA283 and PA284 (lanes 2 and 3) and the downstream region at 540bp amplified by PA301 and PA302 downstream region at 540bp (lanes 4 and 5). Lane 1 represents the New England BioLabs 2-log ladder.

The two regions amplified by PA283 and PA284 or PA301 and PA302 were excised from the gel and purified in order to yield a clean product with which to construct the full deletion fragment. Primers PA284 and PA301 have an overlapping tag which allowed the two gene fragments to be combined via Splicing by Overlap Extension (SOE). This SOE tag was added onto the fragment in the initial PCR reactions. When the two fragments were spliced together, a 990bp fragment was generated. This product was run on a gel, excised, and purified. The purified product was then used in a successive PCR reaction to amplify the product before TOPO cloning. Once this product was verified by a gel it was purified and ready for cloning.

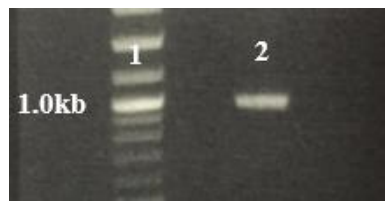


Figure 6: Confirmation of the successful construction of $\Delta potE$ fragments. Lane 2 represents the successful splicing of the upstream and downstream regions of $\Delta potE$ to generate a 990bp fragment. Lane 1 represents the New England BioLabs 2-log ladder.

This product was purified and adenines were added to the 3' ends of the fragment to allow for pCR2.1 TOPO cloning. After the fragment was in pCR2.1, it was electroporated into DH5 α , plated on LB agar plates containing 100 μ g/mL of ampicillin, which had been coated in X-gal for a blue/white screening and, and seven white colonies were tested for the presence of the insert using colony PCR.



Figure 7: Confirmation of the $\Delta potE$ insert in pCR2.1. Seven isolated colonies were chosen with blue/white screening for colony PCR. Colony PCR was performed with primers PA283 and PA302 to verify the presence of the insert. Lane 1: New England BioLabs 2-log ladder, Lane 2: colony 1, Lane 3: colony 4, Lane 4: colony 7, Lane 5: colony 9, Lane 6: colony 11, Lane 7: colony 12, Lane 8: colony 18. All colonies were positive for the 990bp insert.

Colonies 1, 9, and 11 were selected and plasmids were isolated. The plasmids were sent for sequencing and sequence data was verified with VectorNTI to ensure the correct insert, as described in the methods. Colony 1 (AK523) had the correct insert as verified by sequencing. The pCR2.1:: $\Delta potE$ plasmid isolated from AK523 was digested with XhoI and SpeI to release the insert. The digest was separated on an agarose gel and the insert was excised and purified. The insert then was ligated into pWM91 which had also been digested with XhoI and SpeI and purified at the same time (Figure 5).

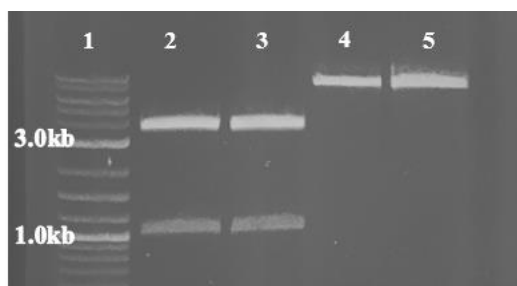


Figure 8: Gel electrophoresis of digested pCR2.1:: $\Delta potE$ and pWM91. In order to ligate $\Delta potE$ into pWM91; pCR2.1:: $\Delta potE$ and pWM91 were digested with XhoI and SpeI. Lane 1 represents with New England BioLabs 2-log DNA ladder, Lanes 2 and 3 represent digested pCR2.1:: $\Delta potE$, Lanes 4 and 5 represent digested pWM91.

After ligation, pWM91:: $\Delta potE$ was then electroporated into *E. coli* DH5 α pir. The successful insertion was verified by colony PCR and then the plasmid was isolated and

electroporated into *E. coli* SM10 λ pir. *E. coli* SM10 λ pir is a conjugating strain and was used for conjugation with *V. cholerae*. The colonies resistant to streptomycin but susceptible to ampicillin were screened via a colony PCR to confirm the presence of the $\Delta potE$ insert (Figure 9). Three colonies were positive for the $\Delta potE$ insert as indicated by a band on the agarose gel at 990bp.

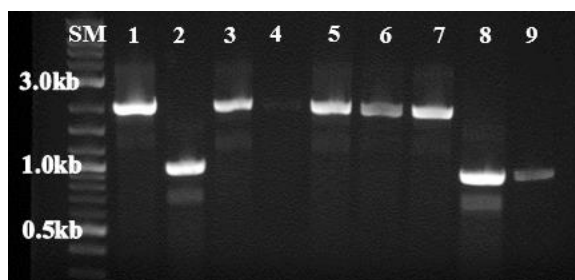


Figure 9: Colony PCR depicting the successful homologous recombination and presence of the $\Delta potE$ insert into PW357 *V. cholerae*. Nine colonies resistant to streptomycin but sensitive to ampicillin were screened via colony PCR. Colonies 2, 8, and 9 were positive for successful insert of $\Delta potE$. SM indicates the size marker, in this case New England BioLabs 2-log ladder. Lane 1 represents colony 1, lane 2 represents colony 2, lane 3 represents colony 3, lane 4 represents colony 4, lane 5 represents colony 5, lane 6 represents colony 6, lane 7 represents colony 7, lane 8 represents colony 8, and lane 9 represents colony 9.

Construction of the $\Delta speB$ single mutant.

The same process used to delete *potE* was repeated for the generation of the $\Delta speB$ mutant. For the $\Delta speB$ mutant regions upstream and downstream of the *speB* gene were amplified using primers PA307, PA308, PA309, and PA310. PA307 and PA308 were used to generate the 297bp upstream fragment while PA309 and PA310 were used to generate the 405bp downstream fragment. PA308 and PA309 had the engineered SOE tag, which was used to fuse the fragments by SOE in a PCR reaction (Figure 10). After the 717bp fragment was fused, it was amplified with PA307 and PA310 by a further PCR reaction.

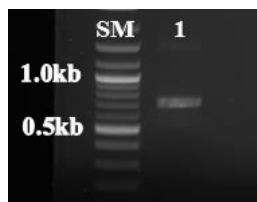


Figure 10: Confirmation of the successful construction of the $\Delta speB$ fragment. This image represents the successful construction of the $\Delta speB$ fragment. SM represents the New England BioLabs 2-log ladder. Lane 1 represents the SOE joining of the upstream and downstream fragments of $\Delta speB$.

Once the fragment was purified, adenines were added to the 3' end to allow for pCR2.1 TOPO cloning. The pCR2.1:: $\Delta speB$ fragment was then electroporated into *E. coli* DH5 α and plated on LB agar plates with 100 μ g/mL ampicillin coated with X-gal. Through a blue/white screening, white colonies were chosen for verification of the $\Delta speB$ insert with a colony PCR. This plasmid was then isolated from colonies 1, 7, and 9, and sent for sequencing.

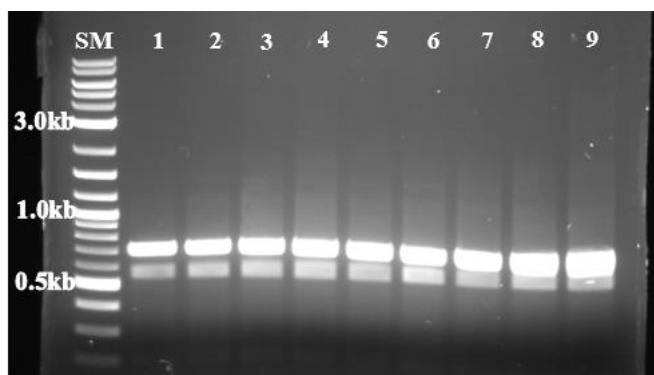


Figure 11: Gel Electrophoresis depicting the successful construction of pCR2.1:: $\Delta speB$ and transformation into *E. coli* DH5 α . Nine isolated DH5 α colonies were chosen by blue/white screening for colony PCR. The colony PCR was performed with PA307 and PA310. SM represents the New England BioLabs 2-log DNA ladder, lanes 1-9 represent colonies 1-9. All colonies had the correct sized insert.

After sequence verification, the plasmid was digested with XhoI and SpeI to release the $\Delta speB$ insert. The insert was then ligated into pWM91 and electroporated into *E. coli* DH5 α pir. Successful insertion was verified with a colony PCR. The plasmid was then removed from *E.*

coli DH5 α pir and electroporated into *E. coli* SM10 λ pir. *E. coli* SM10 λ pir can conjugate with *V. cholerae* PW357. After conjugation, successful homologous recombination was verified with a colony PCR of colonies susceptible to ampicillin but resistant to streptomycin. Colonies 3, 5, and 9 were chosen for further study.

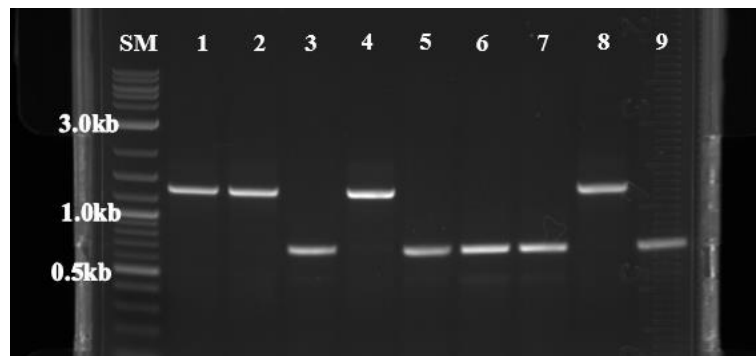


Figure 12: Colony PCR depicting the successful homologous recombination of the $\Delta speB$ insert in PW357 *Vibrio cholerae*. Nine colonies resistant to streptomycin but sensitive to ampicillin were screened via colony PCR. SM indicates the size marker, in this case New England BioLabs 2-log ladder. Lanes 1-9 are representative of colonies 1-9. Colonies 3, 5, 6, 7, and 9 were positive for successful insert of $\Delta speB$.

Construction of the $\Delta speC$ single mutant.

The $\Delta speC$ mutant was constructed in the same way as the $\Delta speB$ and $\Delta potE$ mutants, as described in the methods and above. Regions upstream and downstream of the *speC* gene were amplified via PCR. PA317 and PA318 were used to amplify the 408bp upstream region while PA319 and PA320 were used to amplify the 440bp downstream region. The fragments were spliced together using overlapping tags engineered onto PA318 and PA319. The completed fragment was 863bp long. Gel electrophoresis was used to confirm the successful construction of the fragment. After purification, the fragment was put into the thermocycler to incubate for addition adenines to the 3' ends of the fragment. The added adenines allowed the fragment to be cloned into a pCR2.1 TOPO plasmid. This plasmid was then electroporated into *E. coli* DH5 α .

Colonies were checked via a blue/white screen and presence of the plasmid was verified by colony PCR.

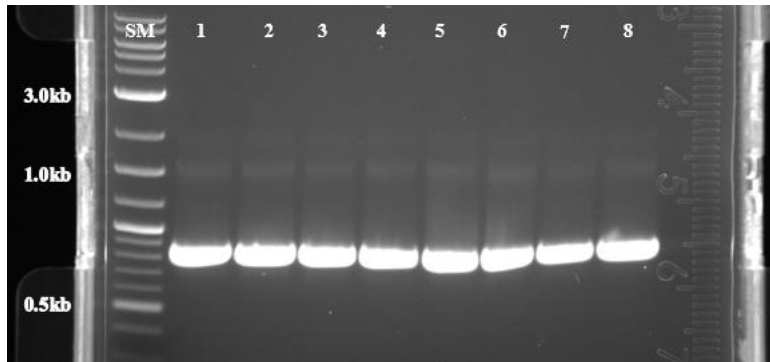


Figure 13: Colony PCR depicting the successful homologous recombination of the of $\Delta speC$ insert in PW357 *Vibrio cholerae*. Nine colonies resistant to streptomycin but sensitive to ampicillin were screened via colony PCR. SM indicates the size marker, in this case New England BioLabs 2-log ladder. Lanes 1-8 are representative of colonies 1-8. All colonies were positive for the $\Delta speC$ insert.

The plasmid was isolated and the sequence of the insert was verified. The pCR.2.1:: $\Delta speC$ was then digested with XhoI and SpeI to isolate the $\Delta speC$ insert, the insert was then ligated into pWM91. pWM91:: $\Delta speC$ was then electroporated into *E. coli* DH5 α pir. The successful insertion was then verified by colony PCR and the plasmid was removed and further electroporated into *E. coli* SM10 λ pir. *E. coli* SM10 λ pir was used for conjugation with *V. cholerae* PW357. After conjugation, colonies were tested for homologous recombination. Colonies that were resistant to streptomycin but susceptible to ampicillin were checked with a colony PCR to confirm the presence of the $\Delta speC$ insert.

Construction of the $\Delta speB\Delta potE$ double mutant.

V. cholerae can both synthesize and transport putrescine. A strain devoid of the only known or proposed transport mechanism and one of the two synthesis pathways was generated. A double mutant was generated by crossing *E. coli* SM10 λ pir containing the pWM91:: $\Delta speB$ plasmid

(AK600) into *V. cholerae* $\Delta potE$ (AK536). Colonies resistant to streptomycin and sensitive to ampicillin were screened with a colony PCR to confirm the presence of the $\Delta speB$ insert. Nine colonies were tested for the presence of the insert. Eight colonies were positive for the insert. Colonies 2, 5, and 7 were chosen for further research.

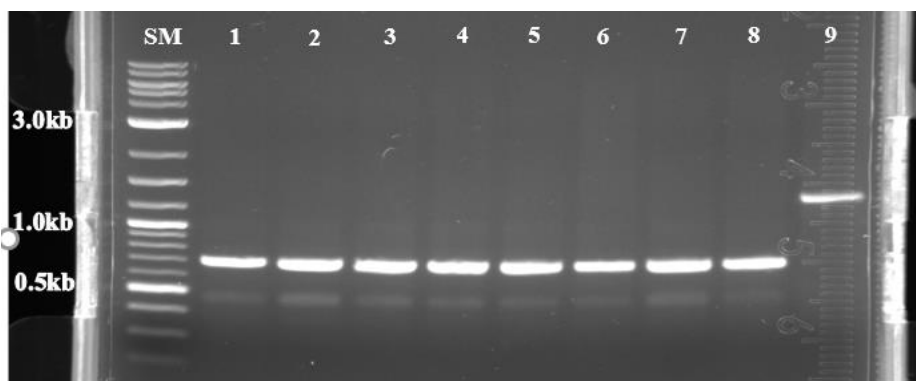


Figure 14: Confirmation of the successful creation of the $\Delta speB\Delta potE$ mutant. Nine colonies were checked for the successful insertion of the $\Delta speB$ insert into the AK536 $\Delta potE$ mutant. PA307 and PA310 were used to check for the insert. SM represents the New England BioLabs 2-log DNA marker. Lanes 1-9 represent colonies 1-9. Colonies 1-8 had appropriately sized fragments which indicated the $\Delta speB$ insert.

Under normal growth conditions there is no difference in the polyamine levels in $\Delta potE$ and wild type *V. cholerae*.

In this research, I sought to characterize the activity of the putative putrescine/ornithine antiporter known as PotE in *V. cholerae*. Previous research in the Karatan lab has been done on the PotABCD system that is spermidine preferential, this system may also import putrescine but likely with greatly reduced efficiency. In *V. cholerae* PotE is the only known or potential dedicated importer or exporter for putrescine. There are other putrescine transporters in bacteria such as *E. coli*; however, a genomic analysis has not revealed them in *V. cholerae*. A mutant lacking the *potE* gene was generated. I hypothesized that there would be a decrease in putrescine intracellularly with this mutant. Polyamines were extracted, benzoylated, and analyzed via HPLC

as detailed in the methods. With each HPLC run a 0.1M standard was used to determine the identity of the polyamines, as described in the methods. The mix contained putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). Under normal growth conditions there does not appear to be a significant difference in intracellular polyamine levels between wild type *V. cholerae* (PW357) and $\Delta potE$ *V. cholerae*. There also does not appear to be any significant difference in any other intracellular polyamine levels (Figure 15).

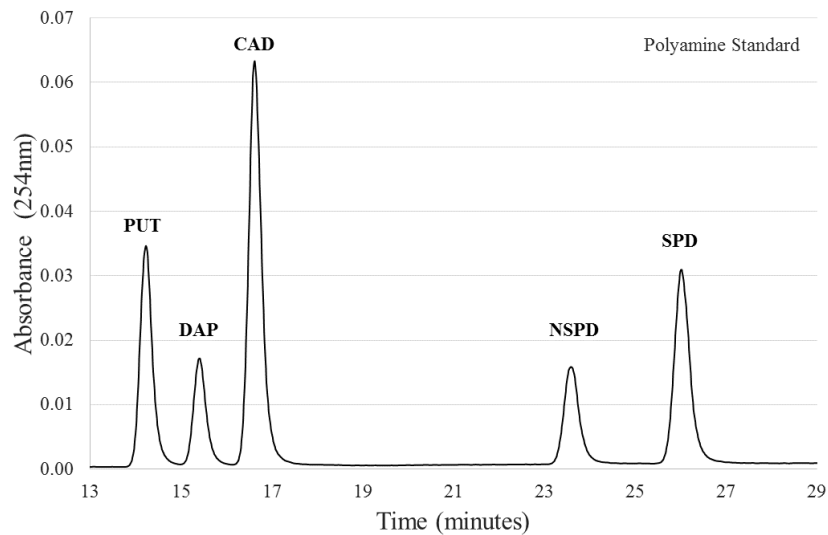
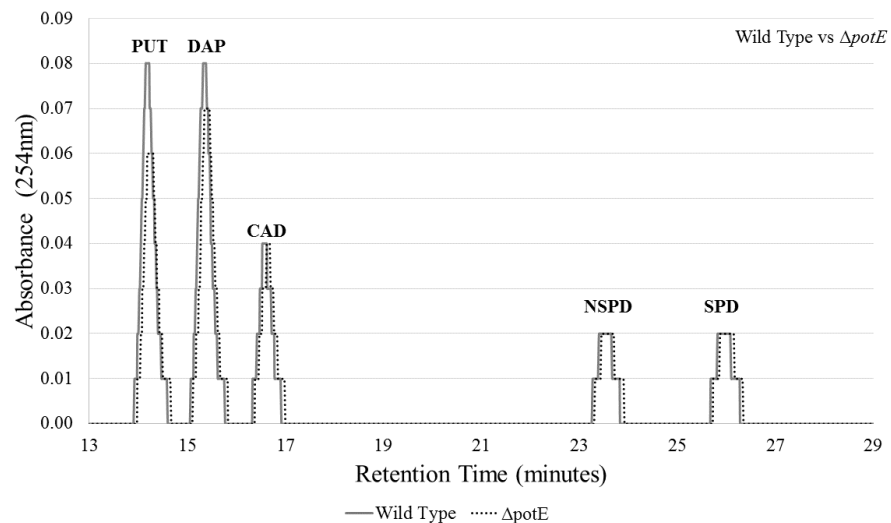
A**B**

Figure 15: Intracellular polyamine levels of wild type *V. cholerae* (PW357) vs the $\Delta potE$ mutant. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to: putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). A) HPLC chromatogram representing the average retention times each polyamine in the standard. B) Overlay of wild type (dark grey line) vs $\Delta potE$ (dotted black line).

Under normal growth conditions there is no difference in the polyamine levels in $\Delta speB$ and wild type *V. cholerae*.

Putrescine is synthesized through two pathways in *V. cholerae*; the first pathway involves the *speB* gene while a second pathway involves the *speC* gene. After seeing no significant difference with the single *potE* mutant I hypothesized that I may see a difference in cellular putrescine levels if I deleted the biosynthesis pathways. I hypothesized that I would see a decrease in intracellular putrescine, especially if *speB* was the primary biosynthesis pathway. Polyamines were extracted, benzoylated, and analyzed via HPLC as detailed in the methods. With each HPLC run, a 0.1M standard was used to determine the identity of the polyamines, as described in the methods. The mix contained putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd) (data not shown). Under normal growth conditions there appears to be a slight decrease in putrescine and cadaverine and a slight increase in diaminopropane although more replicates are needed to determine the significance (Figure 16).

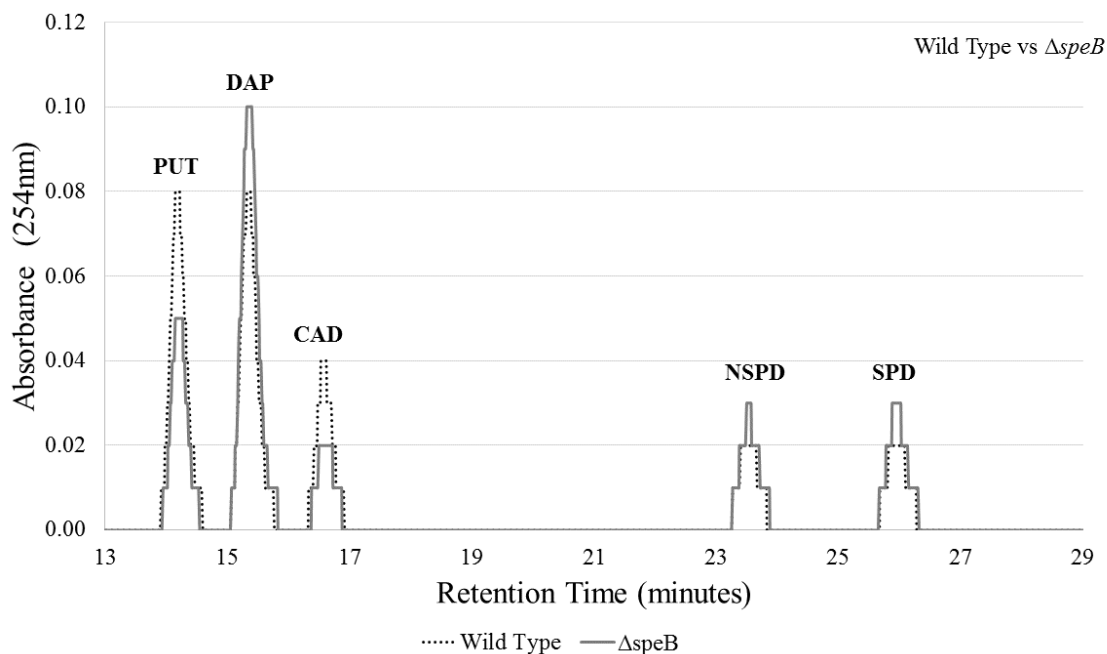


Figure 16: Intracellular polyamine levels of wild type *V. cholerae* (PW357) vs the $\Delta speB$ mutant. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to: putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). This is an overlay of wild type (dotted black line) vs $\Delta speB$ (dark grey line).

Ornithine slightly decreases intracellular levels of cadaverine while increasing intracellular levels of putrescine in a PotE-dependent manner.

Next, I decided to look at the influence of ornithine on the function of PotE. In other bacteria, PotE is a known putrescine/ornithine antiporter. PotE can function by uptaking ornithine while exporting putrescine. Therefore, adding ornithine to the media could induce the transporter. I also decided to use a lower pH as there is evidence from other bacteria that PotE may be upregulated at low pH. I hypothesized that at a low pH with additional ornithine that wild type *V. cholerae* will export putrescine; this export will not be mirrored in the $\Delta potE$ mutant.

Polyamines were extracted, benzoylated, and analyzed via HPLC as detailed in the methods. With each HPLC run a 0.1M standard was used to determine the identity of the polyamines, as described in the methods (data not shown). The mix contained putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). Growth at pH of 5.5 increases the intracellular level of cadaverine of both wild type and the $\Delta potE$ mutant grown with or without ornithine (Figure 17). This was expected as cadaverine is known to be used by other bacteria in order to deal with low pH. Additionally, there is a slight increase in putrescine in the wild-type strain when grown with ornithine at pH 5.5 that is not mirrored in the $\Delta potE$ strain, this increase has been observed in two replicates. This is consistent hypothesis/model that PotE is a putrescine/ornithine antiporter and the wild type is able to import ornithine and degrade it into putrescine.

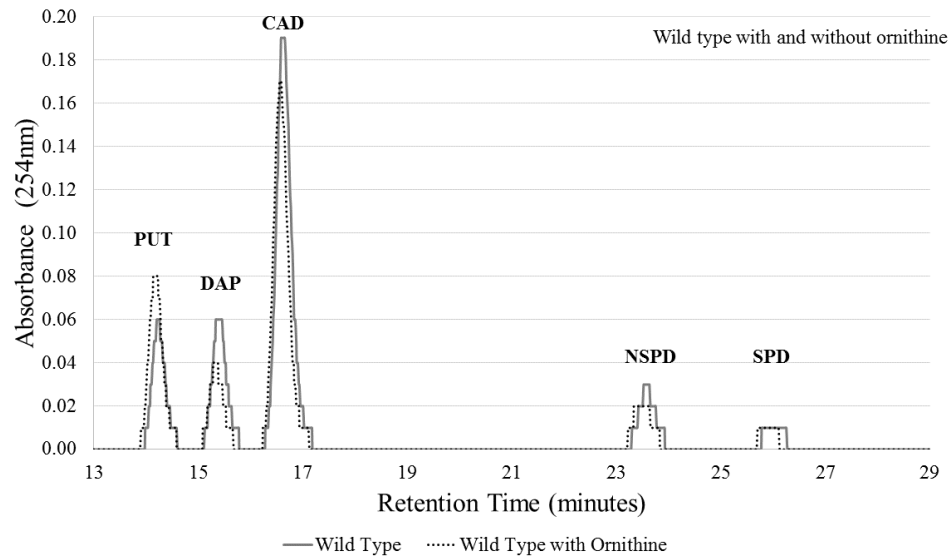
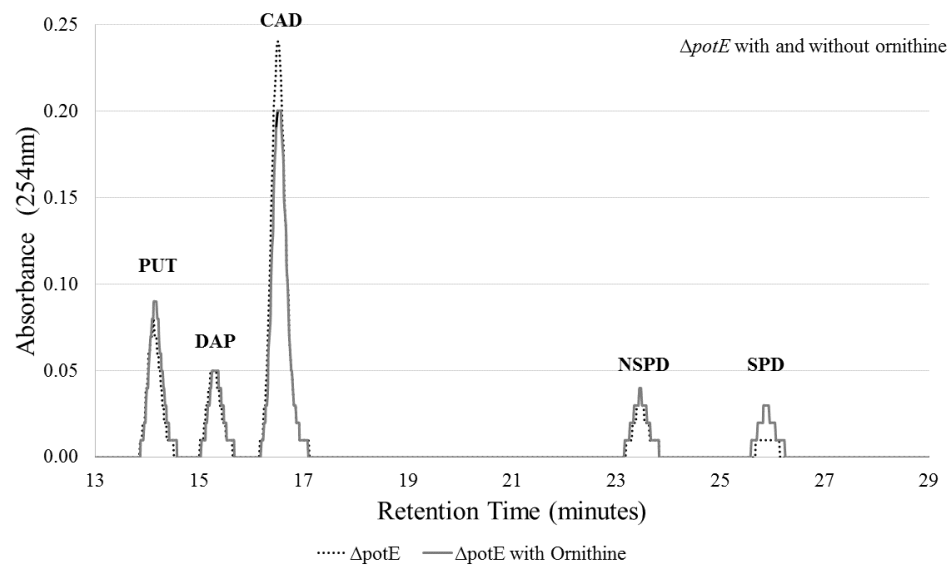
A**B**

Figure 17: Growth at low pH with ornithine increases levels of putrescine in wild type PW357 but not in $\Delta potE$ mutants. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to: putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). A) Wild type grown at pH 5.5 with and without ornithine. B) $\Delta potE$ grown at pH 5.5 with and without ornithine. This is representative of two replicates.

Ornithine in the growth media increases putrescine levels in the media of wild-type cells in a PotE-dependent manner

As observed above, growth at low pH with 5mM of additional ornithine has an effect on cellular putrescine and cadaverine levels of wild-type *V. cholerae* but not of $\Delta potE$ *V. cholerae*.

With those results, I wanted to look at the polyamine levels in the media at the same time point that was used for cellular extract. I hypothesized that there would be more putrescine in the growth media of the wild-type *V. cholerae* when grown in the presence of ornithine.

Additionally, if PotE is a putrescine/ornithine antiporter then there should be no more putrescine in the media of the $\Delta potE$ strain when growth is supplemented with ornithine than without ornithine supplementation. After pelleting the cells, I saved 1mL of growth media and extracted polyamines as described in the methods. Under these growth conditions there was a significant increase both putrescine and cadaverine levels in the growth media of wild-type *V. cholerae*. The increase in wild type cadaverine was around threefold. There was no change in any polyamine level observed in the $\Delta potE$ mutant; however, the cadaverine peak was strikingly high (Figure 18). In the wild type without ornithine, the cadaverine peak is much smaller than either $\Delta potE$ treatment, possibly indicating that if the function of PotE is inhibited the cell will put out cadaverine to deal with what it perceives is a stressful situation. Conversely, the PotE transporter could also be used to transport cadaverine into the media since the molecules are so similar. This data, taken with the above intracellular data is consistent with the hypothesis that PotE functions as a putrescine/ornithine antiporter under the tested growth conditions.

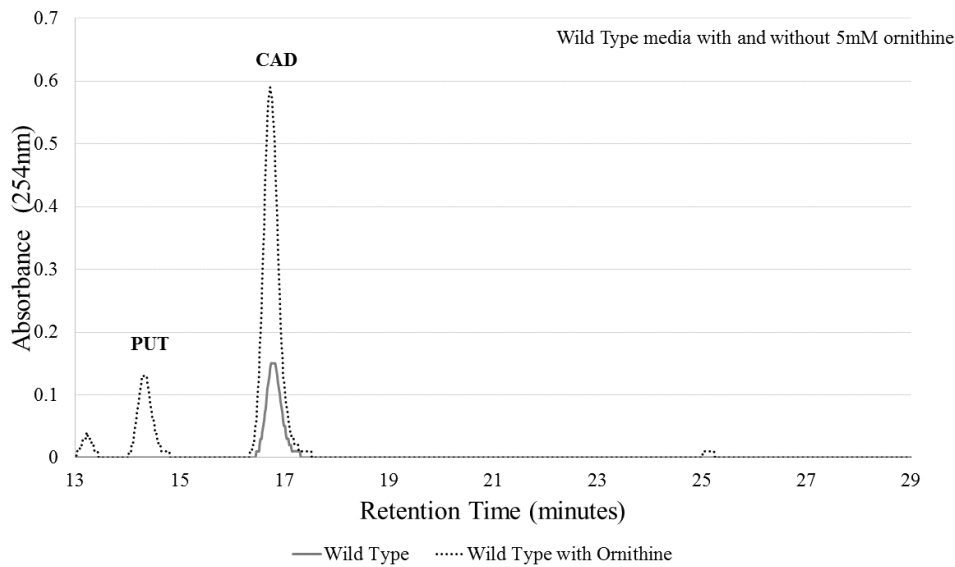
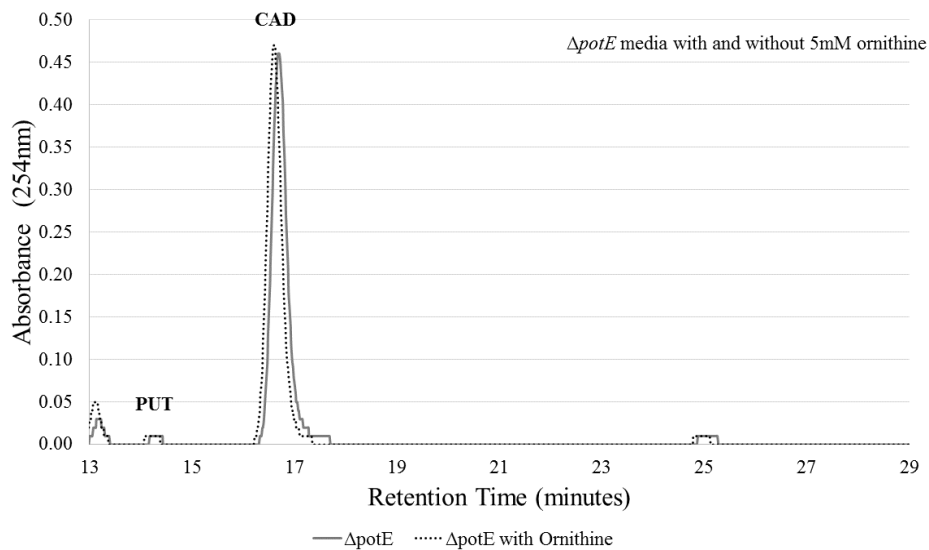
A**B**

Figure 18: Growth at low pH with ornithine increases levels of putrescine and cadaverine in the media of wild type PW357 but not in the media of $\Delta potE$ mutants. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to: putrescine (PUT) or cadaverine (CAD). A) Media of wild type grown at pH 5.5 with and without ornithine. B) Media of $\Delta potE$ grown at pH 5.5 with and without ornithine.

DFMO, an ornithine decarboxylase inhibitor, increases intracellular levels of cadaverine in wild-type and mutant *V. cholerae*.

I was interested in inhibiting both putrescine biosynthesis pathways. One pathway, *speC*, can be inhibited chemically with difluoromethylornithine (DFMO). DFMO is an irreversible chemical inhibitor of ornithine decarboxylase, which is encoded for by the *speC* gene. I hypothesized that DFMO may cause a change in intracellular putrescine levels. Polyamines were extracted, benzoylated, and analyzed via HPLC as detailed in the methods. Under normal culture conditions DFMO's presence in the media at 10mM dramatically increases the cadaverine concentration within the cell, oftentimes by at least double (Figure 19 A-D). The increase in cadaverine was not anticipated as the growth media used in this experiment was not acidic. This effect is seen in wild type PW37, $\Delta potE$ mutant, $\Delta speB$ mutant, and the $\Delta spe\Delta BpotE$ mutant. There is also a slight decrease in intracellular putrescine in all of the mutant strains as compared to wild type. Cadaverine has been shown in other bacteria to be used in mediation of adverse growth conditions, especially low pH conditions. The increase in cadaverine could indicate that the ornithine decarboxylase pathway is also in some way involved with mediating adverse growth conditions and when ornithine decarboxylase is impaired this serves as a signal to the cell that there is a need for excess cadaverine or some sort of cautious growth phenotype. There is a potential that PotE or the ornithine to putrescine biosynthesis pathway in some way also aids cells in dealing with low pH; this is supported by the findings of increased cadaverine when ornithine decarboxylase is impaired as well as the findings in *E. coli* that indicate the function of PotE may at least in part be affected by the pH of the bacterium's surroundings.

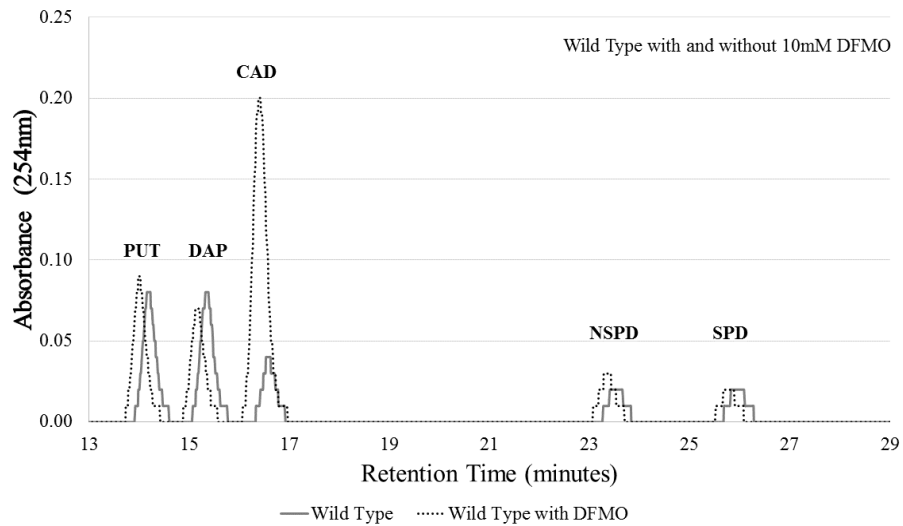


Figure 19 A: DFMO is responsible for the intracellular increase of cadaverine in wild type and mutant *V. cholerae*. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to. Putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). 19 A Represents the change in wild type PW357 *V. cholerae* intracellular polyamine levels in response to DFMO.

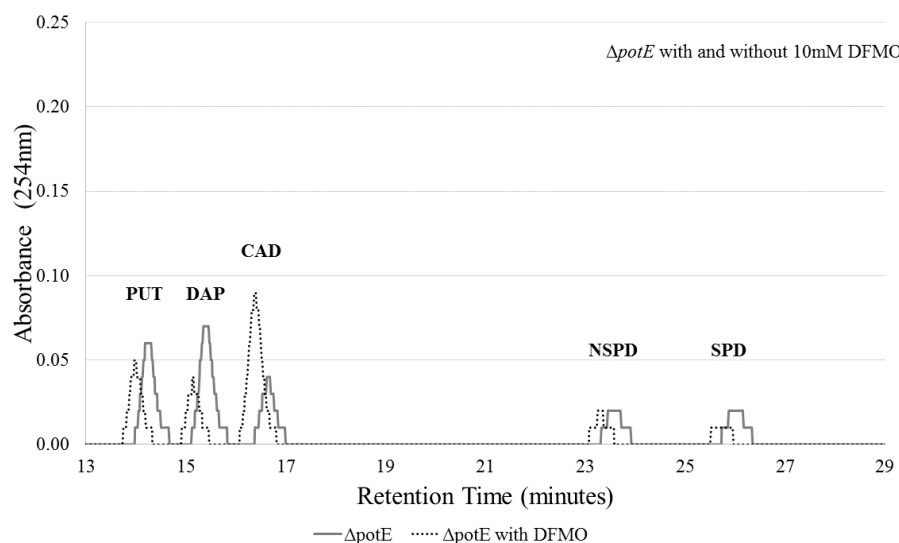


Figure 19 B: DFMO is responsible for the intracellular increase of cadaverine in wild type and mutant *V. cholerae*. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to. Putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). 19 B Represents the change in $\Delta potE$ *V. cholerae* intracellular polyamine levels in response to DFMO.

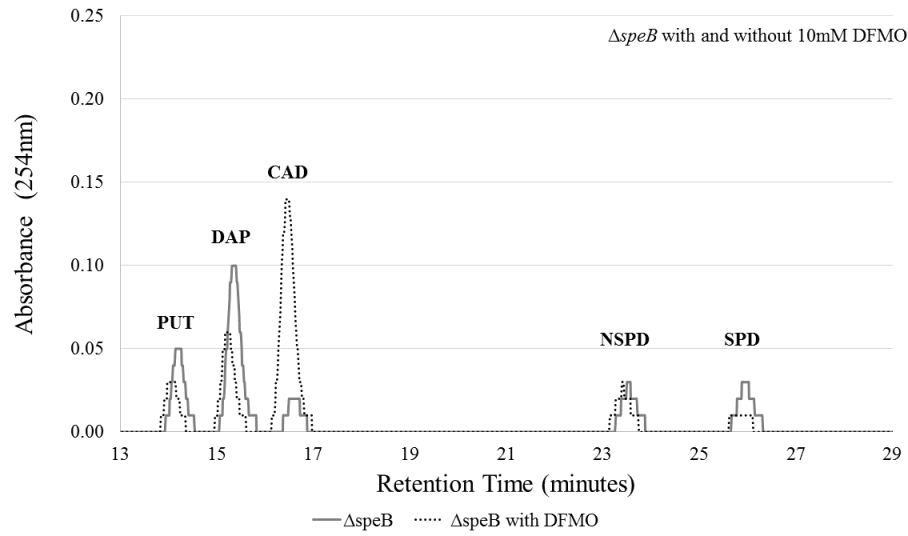


Figure 19 C: DFMO is responsible for the intracellular increase of cadaverine in wild type and mutant *V. cholerae*. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to. Putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). 19 C Represents the change in $\Delta speB$ *V. cholerae* intracellular polyamine levels in response to DFMO.

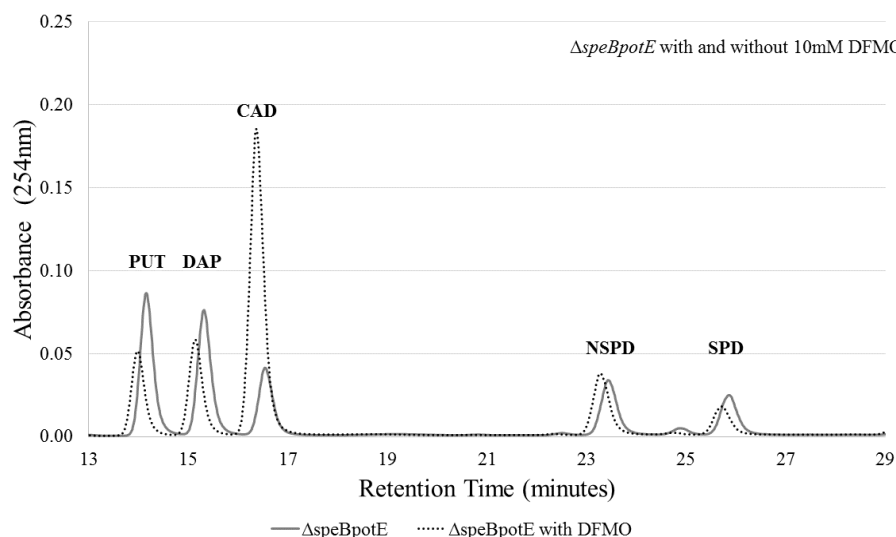


Figure 19 D: DFMO is responsible for the intracellular increase of cadaverine in wild type and mutant *V. cholerae*. For clarity and simplicity, only data collected between 13 and 29 minutes was plotted as that was the range of target polyamines. Peaks are labeled as to which polyamine they correlate to. Putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), and spermidine (SPD). 19 D Represents the change in $\Delta speB\Delta potE$ *V. cholerae* intracellular polyamine levels in response to DFMO.

Under normal growth conditions there is no difference in biofilm formation between $\Delta potE$ and wildtype strains; however, DFMO causes an increase in overall biofilm formation.

DFMO is an irreversible inhibitor of ornithine decarboxylase. Previous research has shown that polyamines can have an effect on biofilms, and I was interested in seeing the effect that an inhibitor of polyamine biosynthesis may have on biofilm formation. Biofilm formation of the $\Delta potE$ and wild-type strains was compared for this experiment. Taking each condition individually, there is not a significant difference between the two strains (Figure 20). This indicates that under normal growth conditions either PotE is not active or its activity does not significantly affect biofilm formation. However, comparing biofilm formation between conditions reveals that there is an increase in biofilm formation when DFMO is added to growth media of wild type or $\Delta potE$ *V. cholerae*. Biofilms are considered a protective life state for the

majority of bacteria, and taken with the above HPLC data this further supports the idea that inhibition of the ornithine decarboxylase biosynthesis pathway in some way indicates to the cell that it is in a stressful situation and may need to protect itself from its environment.

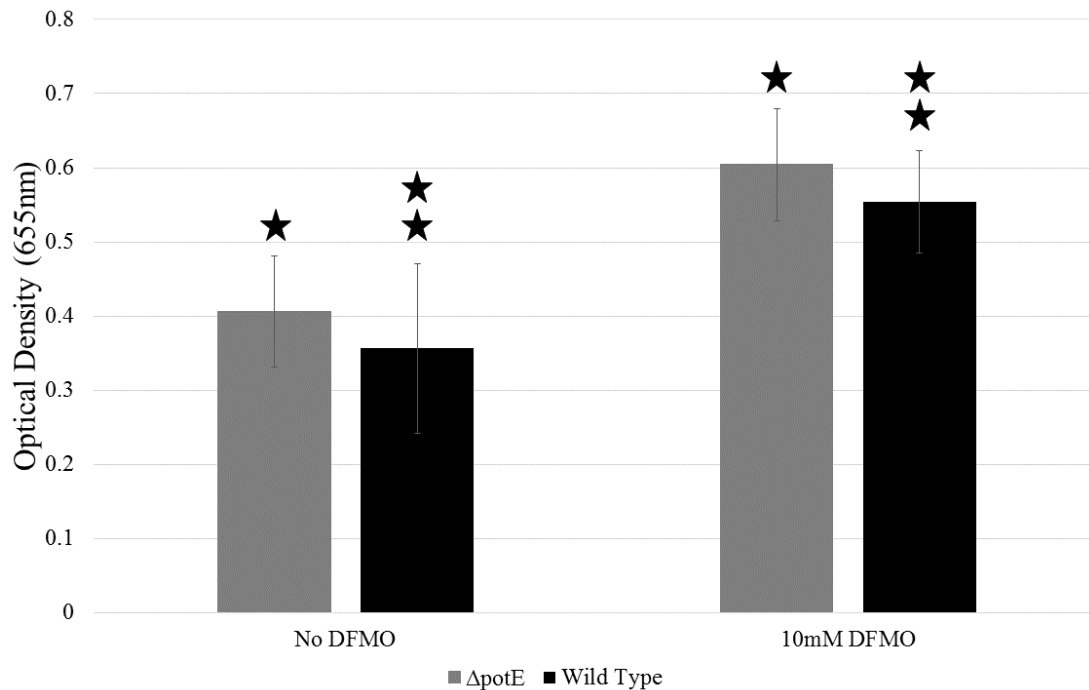


Figure 20: Growth with DFMO increases overall biofilm formation. Biofilm formation of the *ΔpotE* and wild type *V. cholerae*. This chart is an average of nine biological replicates done on three separate days. This trend is also seen in multiple replicates where DFMO is not used. Biofilms were grown for 18 hours in LB media and the error bars are representative of standard deviation. This chart represents optical density of biofilms grown with and without DFMO. P value indicated by a single star for *ΔpotE* with no DFMO vs the 10mM concentration is 1.2×10^{-5} . P value indicated with a double star for the wild type with no DFMO vs the 10mM concentration is 6.4×10^{-4} .

Under tested growth conditions there is no difference in biofilm formation between *ΔspeB* and wildtype.

Each mutant was tested against wildtype for its ability to form a biofilm under defined growth conditions. After seeing a pH effect under certain conditions (Figure 23), I chose to standardize the pH and use an acidic pH and a neutral pH. I hypothesized that under tested

growth conditions if $\Delta speB$ was the primary putrescine biosynthesis pathway then the mutant would form lower biofilm than the wildtype, especially in acidic pH where putrescine could be acting as a mechanism to deal with low pH environments. Comparing the 5.5 pH condition to the 7.5 pH condition, it appears that bacteria grown in 7.5 pH media form higher amounts of biofilm overall (Figure 21). However, there is no significant difference in biofilm formation between the two strains. The lack of significant difference could be attributed to *speC* being the primary biosynthesis pathway in *V. cholerae* or due to the fact that there is still one functioning biosynthesis pathway in the $\Delta speB$ mutant. This pathway may also not normally be active at the tested growth conditions.

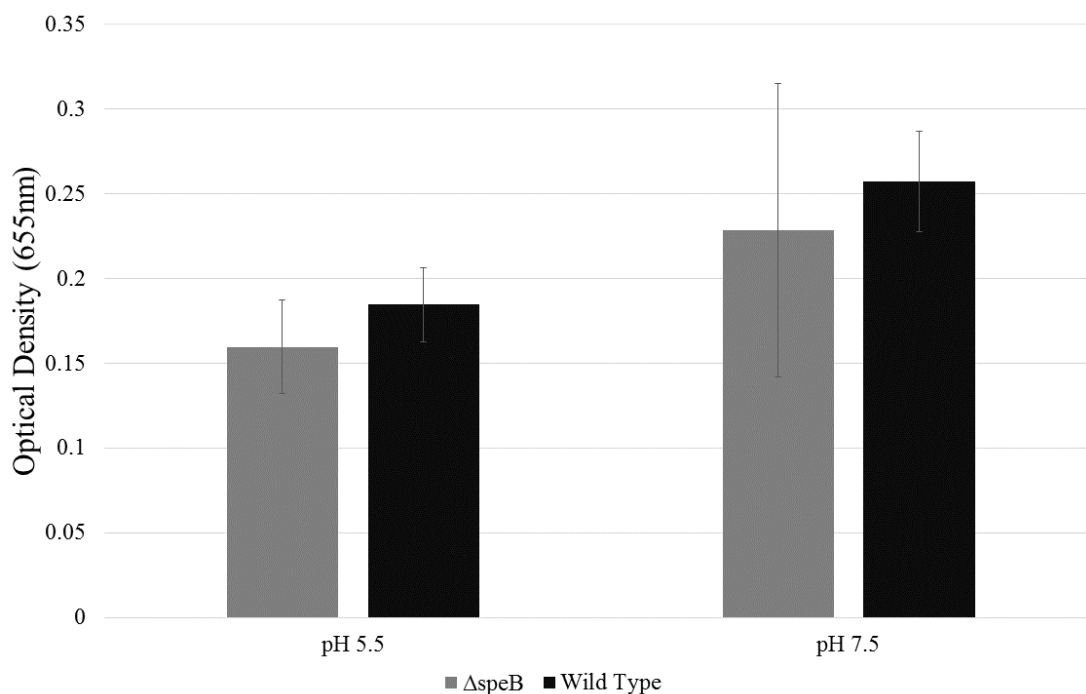


Figure 21: There is no significant difference between wild type and $\Delta speB$ biofilm formation. Biofilm formation of the $\Delta speB$ and wild type *V. cholerae*. This chart is representative of two biological replicates done on two separate days. This trend was also observed in two 18 growth hour biological replicates. Biofilms were grown for 24 hours in LB media and the error bars are representative of standard deviation. This chart represents optical density of biofilms grown at pH 5.5 and 7.5.

Under tested growth conditions there is no difference in biofilm formation between $\Delta speB\Delta potE$ and wild type.

A double mutant was constructed that had genetic defects in both the *speB* and *potE* genes. In order to best study the biosynthesis and transport pathways and their importance to biofilm formation in *V. cholerae*, I needed a way to inhibit each pathway independently. For this biofilm research I used DFMO to inhibit the product of the *speC* gene; this should render both biosynthesis pathways and the transporter non-functional when the $\Delta speB\Delta potE$ strain is grown with DFMO. I hypothesized that if the biosynthesis or transport pathways were important under the tested growth conditions that I would see a significant difference in biofilm formation when compared to wildtype. Overall the biofilms grown at pH 7.5 formed at higher levels than those at pH 5.5. The biofilms formed at pH 7.5 grown in the presence of DFMO were higher than those without, which is consistent with other results (Figure 20). Biofilms grown at pH 5.5 did not seem to increase in the presence of DFMO like those grown at pH 7.5. There was no significant difference observed between strains within a set condition. DFMO in some way works to increase biofilm formation when added to growth media, this may support other data that points to the ornithine/putrescine antiporter of PotE either directly being involved in biofilm mediation or indirectly being involved via the ornithine decarboxylation pathway or an unknown signaling pathway (Figure 22).

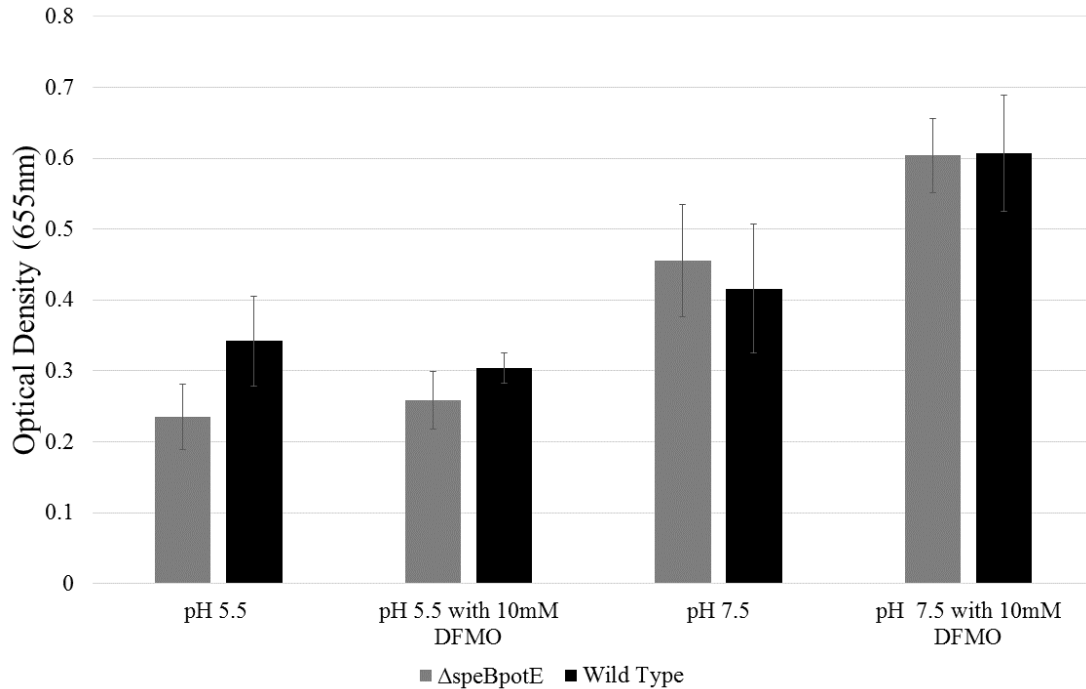


Figure 22: There is no significant difference between wild type and $\Delta speBpotE$ biofilm formation. Biofilm formation of the $\Delta speBpotE$ and wild type *V. cholerae*. This chart is representative of two biological replicates done on two separate days. This trend was also observed in two 18 hour biological replicates. Biofilms were grown for 24 hours in LB media and the error bars are representative of standard deviation. This chart represents optical density of biofilms grown at pH 5.5 and 7.5 with and without 10mM ornithine.

Ability to regulate Ornithine acts as a negative regulator for biofilm formation at low pH.

Following the ornithine and low pH HPLC results in which I saw an increase in intracellular and extracellular putrescine in the wild type but not in the $\Delta potE$ (Figure 17, 18), I wanted to investigate the effect that pH and ornithine addition would have on biofilm formation. For this experiment an acidic pH and a neutral pH were used for biofilm growth. Low pH is a non-ideal growth condition for *V. cholerae*, but it is a condition that the bacteria have to deal with throughout its life cycle. Mediation of low pH environments is important for bacterial survival. I hypothesized that the antiporter activity of PotE may provide some sort of protection at low pH leading to lower biofilm formation in the wild type; I also hypothesized that this

would occur either directly through the export of putrescine which may function in a similar way as cadaverine towards balancing pH or indirectly through an unknown signaling system. It was observed that growth in the presence of ornithine does significantly decrease biofilm formation of wildtype when compared to growth without ornithine (Figure 23). Additionally, there is no effect on $\Delta potE$ growth at pH 5.5 with or without ornithine and the effect that ornithine has on wildtype is significantly different than the effect on $\Delta potE$. Growth at pH 7.5 shows no significance between conditions when strains are considered individually. The $\Delta potE$ strain appears to form slightly higher biofilms overall, although this is not significant. From these results we can conclude that under low pH conditions with additional ornithine, a functioning PotE protein acts as a negative regulator of biofilm formation.

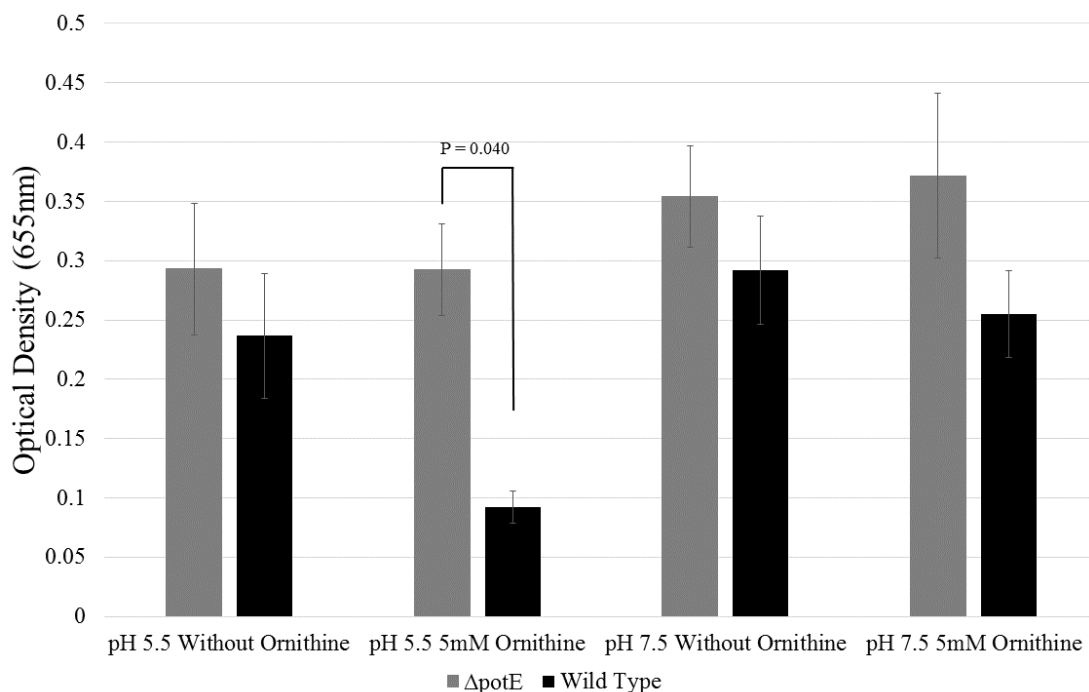


Figure 23: Growth at low pH with 5mM ornithine acts as a negative regulator for biofilm formation. Biofilm formation of the $\Delta potE$ and wild type *V. cholerae* grown at pH 5.5 and pH 7.5 with and without ornithine. This chart is representative of two biological replicates done on two separate days, additional replicates were also performed and the same trend was observed in four biological replicates. Biofilms were grown for 24 hours in LB media and the error bars are representative of standard deviation.

There is no significant difference between $\Delta potE$ and wild type *V. cholerae* biofilm formation over time.

Because biofilm levels appeared to be slightly higher in the $\Delta potE$ mutant at the 24-hour time point, I hypothesized that there may potentially be a difference in biofilm formation between the $\Delta potE$ and wild type strains at a time point not frequently looked at. To determine if there was a difference in biofilm formation over time between $\Delta potE$ mutant and the wild-type *V. cholerae*, multiple replicates were set up and scored every two hours from 4 to 20 hours. In both strains for the first 8 hours, biofilm formation was relatively minimal and there was not a significant difference observed (Figure 20). Around hour 10 it appears that biofilm formation begins to pick up in both strains but this there is also no significant difference. Biofilm formation continues to increase from hours 12-20 but there is no significant difference between strains. Under normal growth conditions there does not appear to be a difference in the rate of formation of biofilm for $\Delta potE$ and wild type strains.

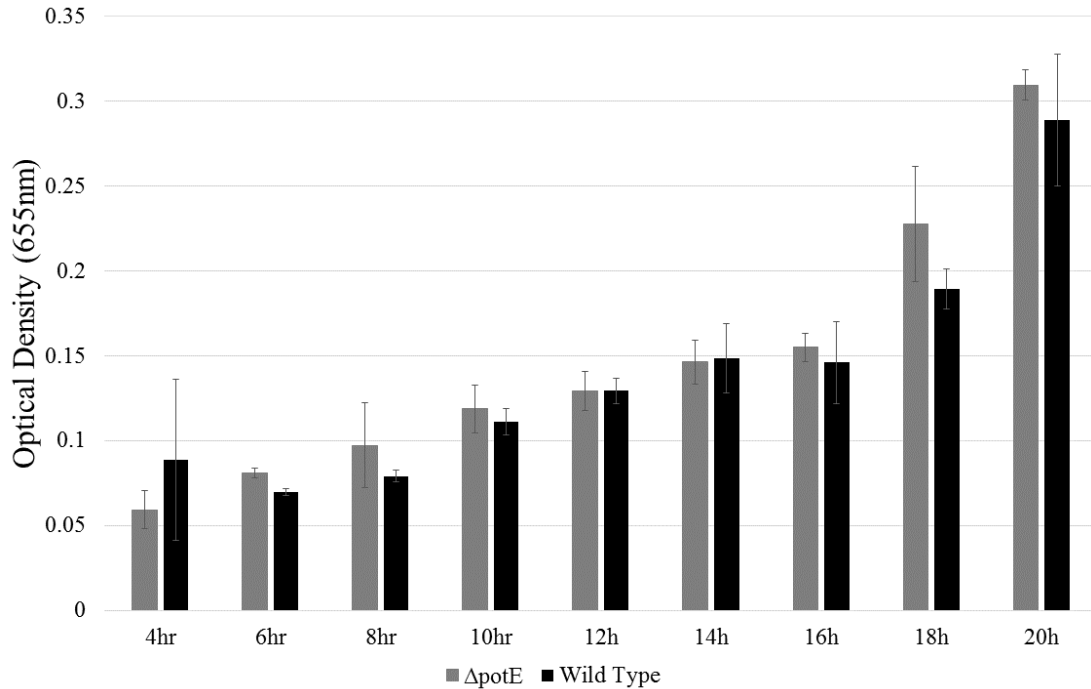


Figure 24: There is no significant difference between wild type and $\Delta potE$ biofilm formation over time. Biofilm formation of the $\Delta potE$ and wild type *V. cholerae* over 20 hours. This is representative of two replicates. Biofilms were grown in LB media and the error bars are representative of standard deviation. This chart represents optical density.

Discussion

The purpose of this study was to characterize the PotE transporter in *V. cholerae* and how PotE affects *V. cholerae* biofilms. To best achieve the original goal, I also investigated the two putrescine biosynthesis pathways in *V. cholerae*. This led me to investigate the role that both putrescine biosynthesis and transport have on biofilm formation in *V. cholerae*. Little research had previously been done on putrescine in *V. cholerae*; however, putrescine is one of the most widespread polyamines. Putrescine is found in most species and the majority are able to synthesize it including *V. cholerae* (Morgan, 1999). In *E. coli* putrescine is synthesized by two pathways and can be imported or exported by the cell with the use of a transporter. Based on previous work in *E. coli* and the conserved genes in *V. cholerae* I hypothesized that PotE is a

putrescine/ornithine antiporter and that putrescine can be synthesized in *V. cholerae* from ornithine or arginine (hypothesized model is illustrated in Figure 25). I was able to successfully show PotE functions as a putrescine/ornithine antiporter, the first putrescine/ornithine antiporter characterized in *V. cholerae*.

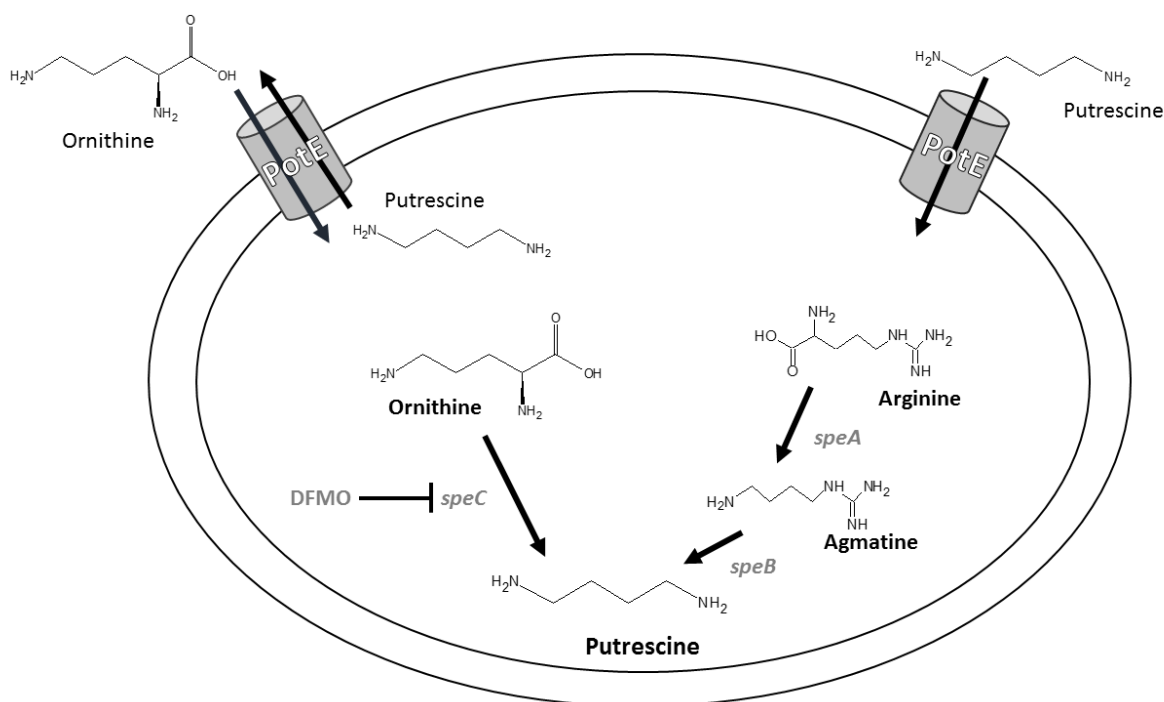


Figure 25: Model of the predicted pathways of putrescine transport and biosynthesis in *V. cholerae*. PotE is the only known or potential transporter of putrescine in *V. cholerae*. There are two pathways of putrescine formation: ornithine to putrescine and arginine to agmatine to putrescine.

Investigation of putrescine synthesis and transport pathways in *V. cholerae*

Previous research in the Karatan lab has used HPLC to quantify the intracellular polyamine levels in *V. cholerae* and has shown that putrescine is one of the main polyamines made by *V. cholerae*. Because of that I wanted to measure the polyamine levels in the mutants $\Delta potE$, $\Delta speB$, $\Delta speB \Delta potE$, as they have mutations for what I propose are the major polyamine transport and one of the major biosynthesis pathways. I initially hypothesized that I would see a

decrease in the putrescine levels within the mutants; however, this was not the case. Under normal growth conditions, there is no observable difference in in any of the mutant strains ($\Delta potE$, $\Delta speB$, $\Delta speB\Delta potE$) when compared to wild type. There is a slight decrease in putrescine levels in some replicates, such as $\Delta speB$ and $\Delta potE$ (Figure 15, Figure 16), but this decrease is not large enough to be ruled out as natural variation and more replicates of these conditions are needed to make a full conclusion if the decrease is because of the mutant or just natural growth variation. This slight decrease may indicate that the genes of interest do perform maintenance of putrescine levels under normal growth conditions but their loss is not significant enough to negatively affect the bacteria. While it is probable that *potE* and *speB* function under normal growth conditions, it has also been shown that the operon *potE* and *speC* belong to is repressed at neutral extracellular pH, which potentially explains the lack of variation under normal growth conditions or normal growth conditions with DFMO (Kashiwagi et al., 1997). Additionally, there is evidence to suggest that the arginine to putrescine pathway is only the preferred pathway when there is excess arginine, possibly explaining why the lack of *speB* is not yielding significant results (Morris & Koffron, 1969)

To inhibit both biosynthesis and the one transport pathway at the same time, I constructed a double $\Delta speB\Delta potE$ mutant and supplemented the growth media with DFMO; DFMO is a specific inhibitor of ornithine decarboxylase, the produce of *speC*, which will irreversibly inhibit the decarboxylation of ornithine into putrescine. When *V. cholerae* was grown with DFMO added to the media, I observed that intercellular levels of cadaverine spiked, oftentimes two or more times greater than levels without DFMO supplementation in all mutant and wild-type strains. In all of the mutants tested, levels of putrescine and diaminopropane appeared to decline slightly as compared to the mutant strains grown without DFMO. This slight decrease in

putrescine and diaminopropane was not seen in wildtype. It is possible that DFMO does not inhibit *V. cholerae* ornithine decarboxylase. However, this is highly unlikely as DFMO inhibits ornithine decarboxylase of *Vibrio vulnificus*, a related *Vibrio* pathogen (Lee, Michael, Martynowski, Goldsmith, & Phillips, 2007). Because DFMO is known to inhibit the ornithine decarboxylase of another member of the *Vibrio* species, I hypothesized it may inhibit *V. cholerae* as well. It is also possible that under the growth conditions I performed these experiments in the bacteria do not need a high level of putrescine to carry out normal growth functions.; therefore, even small amounts of ornithine decarboxylase remaining in the cell could potentially support the production of putrescine.

Additionally, the DFMO addition is likely to be acting upon a cellular process as indicated by the strikingly high cadaverine levels. Cadaverine is imported into the cell by a transporter similar to PotE known as CadB (Figure 26). CadB is inducible at low pH and is believed to be a mechanism for bacteria, specifically *E. coli*, to respond to acidic conditions (Küper & Jung, 2006; Meng & Bennett, 1992; Watson et al., 1992). In *V. cholerae* and *Vibrio vulnificus*, low pH triggers the production of cadaverine which is then exported to neutralize low environmental pH and protect the cell from acid stress (Kang et al., 2009; Merrell & Camilli, 1999). CadB is also used to protect *V. vulnificus* from superoxide stress at a basic pH (Kang et al., 2009). The results suggest that addition of DFMO indicates to the cell that there is a stressful situation and to deal with that situation it must increase the level of cadaverine produced and exported. HPLC of the media also indicated high levels of cadaverine (data not shown). This could be a side effect of the DFMO itself or it could be that when ornithine decarboxylase is inhibited and the cell can no longer properly deal with the buildup of ornithine it induces excess cadaverine synthesis to help it deal with a perceived stressful situation. Or normally, some

amount of both cadaverine and putrescine need to be synthesized under acidic conditions and the decrease in putrescine is compensated by an increase in cadaverine levels. Due to the similarity of CadB and PotE and the similarity of the putrescine and cadaverine molecules themselves, it is possible that they function in a similar manner to deal with stress to the cell. This data also suggests that the inability to dispose of excess ornithine by converting it to putrescine, which would then be transported out of the cell, is considered by the cell to be a stressful situation. It is possible that inhibition of the function of ornithine decarboxylase could serve to upregulate *cadBA*, which encodes CadB and cadaverine decarboxylase (Soksawatmaekhin et al., 2004). Unless the increase in cadaverine levels is caused solely by the addition of DFMO, which will be resolved with a *speC* mutant, it appears likely that these two pathways are linked in a stress response for *V. cholerae*.

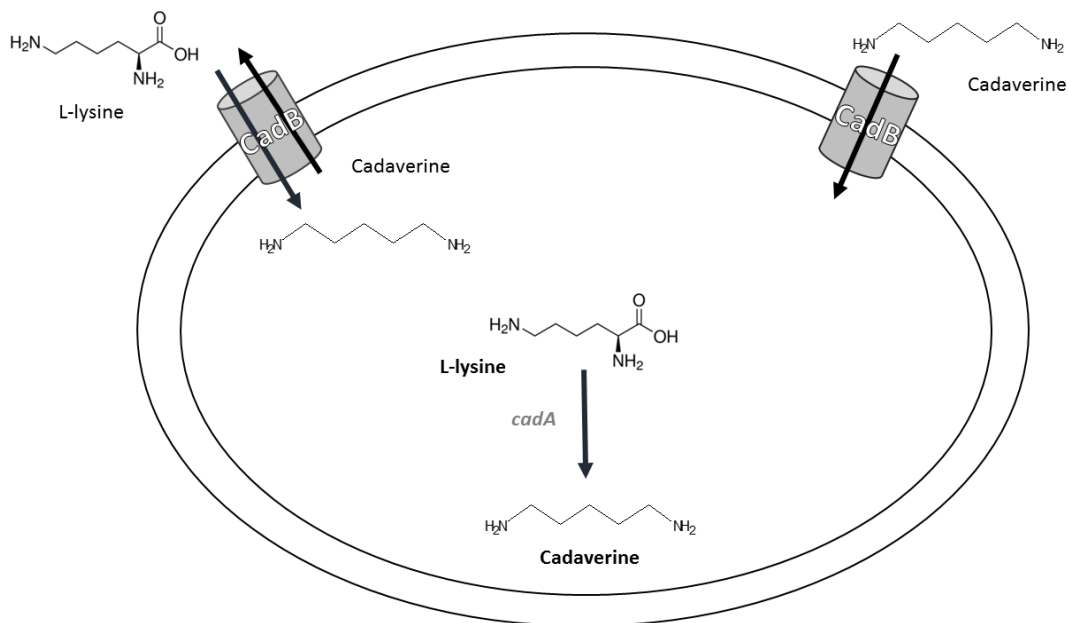


Figure 26: Model of the predicted pathways of cadaverine transport and biosynthesis in *V. cholerae*. CadB is the only known or potential transporter of cadaverine in *V. cholerae*. There is one pathway of cadaverine formation: L-lysine to cadaverine.

I was also able to show that PotE functions as a putrescine/ornithine antiporter in *V. cholerae*. I also showed that PotE is the only protein that has this role as deletion the *potE* gene resulted in no export of putrescine even when the media was supplemented with ornithine. Low pH was chosen for this experiment because in *E. coli* low pH and excess ornithine have been seen to induce *potE* (Igarashi & Kashiwagi, 2010b). Figure 17 shows that growth at low pH in the presence of ornithine decrease the level of putrescine in wild-type cells and that this decrease is not seen in the $\Delta potE$ mutant. More importantly, there is a significant increase in the level of putrescine in the media of wild-type cells grown with ornithine as compared to those without (Figure 18). This increase is not seen in the $\Delta potE$ mutant. Also, adding to the data of a potential link between the CadB and PotE pathways, cadaverine levels in the media increase in the wild type grown with ornithine as compared to the control. Cadaverine levels in the media are also

strikingly high for $\Delta potE$, although they do not change with the presence of ornithine like the wild type levels. This may indicate that inhibition of PotE serves as a signal to the cell that there is a stressful situation it needs to respond to. Since cadaverine is known to deal with acid stress this could indicate that inhibition of PotE indicates to the cell that it is in a stressful or acidic environment. In wild type, this increase in cadaverine could potentially be in response to the buildup of excess ornithine being imported into the cells. This data along with the biofilm data in Figure 23 that shows a decrease in biofilm levels in wild type grown at low pH in media supplemented with ornithine is consistent with the hypothesis that a functional PotE protein is used to transport ornithine into the cell while transporting putrescine out of the cell and that this transport has an effect on cellular physiology at low pH environments. The cell is then able to convert ornithine into putrescine for further use or export. Additionally, other research has shown that during infection the genes for *potE* and *speC* are upregulated a signifying amount in mouse and rabbit intestines (Mandlik et al., 2011). This upregulation is an important finding because it indicates that these genes play a role in the infection cycle of *V. cholerae*. Although more research is needed, this upregulation could be tied to a stress response similar to the one seen here.

These results do not disprove the potential existence of some sort of unidentified retroconversion pathway. For instance, when DFMO is added to *A. tumefaciens* there is a retroconversion pathway that converts spermidine to putrescine (Kim et al., 2015). Similarly, spermidine that is taken up by *V. cholerae* under my experimental conditions could potentially be converted retroconverted to putrescine. In that study the retroconversion was not framed as a way to preserve putrescine levels, however, the possibility exists nonetheless.

The data for biofilm formation of $\Delta potE$, $\Delta speB$, and $\Delta spe\Delta BpotE$ also may indicate the existence of a yet undiscovered secondary importer of putrescine in *V. cholerae*. *E. coli*, for instance, has multiple putrescine transporters including: PotFGHI, PuuP, PlaP, and PotE (Kashiwagi et al., 1991; Kurihara, Suzuki, Oshida, & Benno, 2011; Kurihara et al., 2009; Pistocchi et al., 1993). Of these, only PotE is known to exist in *V. cholerae*. This would also help explain the lack of difference in intracellular putrescine levels in the mutants. Additionally, a known transporter may be able to act as a putrescine importer under certain conditions; for instance, the PotABCD transport system can transport putrescine when it's present at high concentrations instead of its preferred molecule so when the PotE transport system is not working properly it is possible that PotD1 or PotD2 could mediate putrescine uptake, although via HPLC analysis of the media there appear to be very low levels of putrescine in LB broth. This has been considered; however, it is unknown if the transport is relevant or possible in the natural environment. However, we have not ruled out here the existence of a secondary pathway functioning to import putrescine during times of stress.

Effect of putrescine synthesis and transport pathways on *V. cholerae* biofilm formation

Because polyamines such as norspermidine and spermidine have been shown to affect biofilm formation in *V. cholerae*, I wanted determine whether putrescine synthesis and/or transport pathways also have an effect on *V. cholerae*. I found that under normal growth conditions, there was not a significant difference in biofilm formation between the wildtype and the *potE* mutant. This may indicate that that under tested conditions that the bacteria can regulate the levels of polyamines within the cell via biosynthesis and that putrescine transport is not completely necessary, or that putrescine transport does not regulate biofilms. Additionally, LB broth has a low level of putrescine naturally which could inhibit transport. However, even when

excess putrescine was added to the growth media no significant change from wildtype was seen. Also, as mentioned above, the expression of the *potE* gene is repressed at neutral pH. Putrescine could also be synthesized by the cell if its uptake is blocked or environmental conditions signal low levels of putrescine. Additionally, it is not known if *V. cholerae* needs large amounts of putrescine to function under otherwise good growth conditions, therefore we may be seeing no significant change in putrescine levels due to the molecule not being needed.

Since there was no significant difference in biofilm formation in the $\Delta potE$ mutant, I wanted to explore if there would be an impact on biofilm formation when the *speB* gene was deleted. The *speB* gene encodes agmatine uerohydrolase that makes putrescine from agmatine. When the gene was deleted I was unable to detect a significant change in biofilm formation when compared to wild type *V. cholerae*. It is not entirely unexpected to see no significant change from the deletion on the *speB* gene. There is evidence to suggest that this pathway is not the primary pathway of putrescine synthesis in *E. coli* under normal growth conditions. Studies have shown that in *E. coli*, decarboxylation of ornithine to putrescine is the most direct pathway of putrescine synthesis, which may make it the most efficient to use especially when nutrients are limited (Morris & Koffron, 1969; Morris & Pardee, 1966). The biosynthesis pathway of arginine to putrescine is also noncompetitively inhibited by ornithine and by physiological concentrations of putrescine and spermidine (Morris et al., 1970; Satishchandran & Boyle, 1986). If putrescine is not needed by the cell in large amounts, any decrease in the time periods tested may not be enough to stop the inhibition of the arginine to putrescine pathway. Additionally, putrescine itself can repress the transcription of the *speA* gene (Moore & Boyle, 1991). The lack of effect from the deletion of the *speB* gene doesn't completely disprove that *speB* gene or that pathway of putrescine conversion is necessary to *V. cholerae*. Laboratory growth conditions do not match

those found in the natural environment of the bacteria, and this gene could be more valuable under untested environmental conditions.

I wanted to see if a complete inhibition of the putrescine biosynthesis or transport pathways would have a significant phenotypic impact on the cells when compared to wild type. There appeared to be no detrimental effects on the growth of *V. cholerae* biofilms when both the *speB* and *potE* genes were deleted and ornithine decarboxylase was inhibited by DFMO. However, in neutral pH condition we did see an increase in biofilm formation in DFMO conditions, however this was not mirrored in acidic conditions. Additionally, levels of putrescine within the cell were comparable to wildtype in the mutants with DFMO. This does not prove that putrescine is unnecessary to the cell, only that under the tested conditions in a highly nutritious growth medium *Vibrio cholerae* may have little need for excess putrescine beyond what may have already been inside of the cell at the time the DFMO was added.

I wanted to begin testing growth conditions that would be considered non-optimal to the bacterium. One such condition was a low media pH. A pH of 5.5 was chosen because *V. cholerae* did not grow in media with pH lower than 5.5 (data not shown). Additionally, the planktonic cells could reasonably be expected to encounter this pH in their infection cycle as the human caecum has a pH of around 5.7. When bacteria were grown at a low pH with added ornithine I observed that the wildtype strain formed significantly less biofilm than the $\Delta potE$ mutant or the wildtype that was not treated with ornithine. Quantification of extracellular putrescine levels under these conditions showed that the PotE protein serves to import ornithine and export putrescine. Also, this import appears to function to negatively regulate the formation of biofilms. It is unknown if this regulation occurs directly through the antiporter function of PotE or if the regulation is indirect and depends on putrescine, ornithine, ornithine

decarboxylation, or a combination thereof being responsible for the negative regulation. In any case, this allows the cells to remain in a planktonic state for longer than they would otherwise and likely conveys some sort of survival advantage to them by helping them to deal with stressful environmental conditions. This also supports the hypothesis that PotE and putrescine may function in a similar way to CadB and cadaverine when helping the bacteria to deal with low environmental pH.

Future Directions

The deletion of the *speC* gene is needed to fully confirm the results seen by addition of DFMO. Without the full deletion of the *speC* gene we cannot confirm that the heightened biofilm growth when DFMO is added is a side effect of the DFMO or if it is a result of ornithine decarboxylase being inhibited. We also can not fully confirm if the high levels of cadaverine seen with HPLC analysis when DFMO is added to the growth media is directly linked to the inhibition of ornithine decarboxylase or if it is a secondary effect. More replicates are also needed for many of the tested conditions to add statistical power to the results. The role of pH should also be investigated further, including low pH conditions with both added DFMO and ornithine. It could be of interest to do a pH challenge experiment, in which the biofilm is allowed to grow under neutral conditions before the spent media is removed and replaced with a low pH media. This experiment would be of use to test the ability of the biofilms formed by the mutants and wild type to withstand harsh environmental conditions.

For further study, RNA sequencing could be useful to identify the genes that are differentially expressed under the varying growth conditions used in this research. Investigating gene expression patterns could also help us understand what conditions to use in research to best study the effect that putrescine has on *V. cholerae*. RNA-Seq has shown in mouse and rabbit

models, that in infection *potE* and *speC* are upregulated during colonization; this should be further investigated based on my results, potentially by doing colonization experiments with the mutant strains generated here (Mandlik et al., 2011).

Additionally, research in *E. coli* has indicated that the arginine to putrescine is preferential when there is a surplus of arginine in the growth media. This would be useful to research in the future to see if arginine supplementation of the growth media could cause a significant increase in the putrescine levels in the wild type *V. cholerae* as compared to mutant *V. cholerae*.

Conclusions

From this study I was able to conclude that PotE is a putrescine/ornithine antiporter at low pH and that this function may be induced at low pH. These are similar to the findings for the function of PotE in *E. coli*; however, this had never before been verified in *V. cholerae*. This could be an important finding to further understand the role of polyamines in the life cycle of *V. cholerae*. As *V. cholerae* is an aquatic pathogen most often found in brackish water or colonizing the small intestine of a human, it is useful to understand the pathways utilized in the myriad of environmental conditions *V. cholerae* must face. If the availability of excess ornithine at low pH allows the cells to stay in a planktonic state longer it could mean that this gives them more time to disperse to more ideal habitats within the aquatic environment before needing to enter a biofilm state. This could also allow the planktonic state to survive passage through the intestines. While the small intestine has a more ideal pH ranging from 6-7.5 that pH drops to around 5.7 in the caecum, which is near the range of our tested conditions for low pH (Fallingborg, 1999). Being able to move through the intestines without being forced to form a biofilm could provide a significant advantage to *V. cholerae* upon its release into the environment and it could also allow

it to secrete harmful toxins within its host for a longer period of time as *V. cholerae* can only secrete the cholera toxin when in its planktonic state. While the mutant strains constructed in this study did not show significant changes in biofilm formation under normal growth conditions, that could indicate that PotE or putrescine are used more in times of stressful growth conditions than in times of good growth conditions, which mirrors CadB and cadaverine. Additionally, this data further suggests a link between PotE and CadB in *V. cholerae* that warrants further investigation.

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